

# Analytical Profiles of Drug Substances

Volume 18

*Edited by*

**Klaus Florey**

The Squibb Institute for Medical Research  
New Brunswick, New Jersey

*Contributing Editors*

Abdullah A. Al-Badr	Harry G. Brittain
George A. Forcier	Lee T. Grady



ACADEMIC PRESS, INC.

**Harcourt Brace Jovanovich, Publishers**

San Diego New York Berkeley Boston  
London Sydney Tokyo Toronto

## **EDITORIAL BOARD**

Abdullah A. Al-Badr	George A. Forcier
Gerald S. Brenner	Lee T. Grady
Glenn A. Brewer	Eugene L. Inman
Harry G. Brittain	G. Williams Martin
James E. Carter	John E. Zarembo

*Academic Press Rapid Manuscript Reproduction*

This book is printed on acid-free paper. (∞)

COPYRIGHT © 1989 BY ACADEMIC PRESS, INC.

All Rights Reserved.

No part of this publication may be reproduced or transmitted in any form or by any means, electronic or mechanical, including photocopy, recording, or any information storage and retrieval system, without permission in writing from the publisher.

ACADEMIC PRESS, INC.

San Diego, California 92101

*United Kingdom Edition published by*

ACADEMIC PRESS LIMITED

24-28 Oval Road, London NW1 7DX

LIBRARY OF CONGRESS CATALOG CARD NUMBER: 89-659072

International Standard Serial Number: 0099-5428

ISBN 0-12-260818-6 (alk. paper)

PRINTED IN THE UNITED STATES OF AMERICA

89 90 91 92 9 8 7 6 5 4 3 2 1

## **AFFILIATIONS OF EDITORS AND CONTRIBUTORS**

*Ezzat M. Abdel-Moety*, College of Pharmacy, King Saud University,  
Riyadh 11451, Saudi Arabia

*Abdullah A. Al-Badr*, King Saud University, Riyadh 11451, Saudi Arabia

*Hamad A. Al-Khamees*, College of Pharmacy, King Saud University,  
Saudi Arabia

*Abdulrahman M. Al-Obaid*, College of Pharmacy, King Saud University,  
Riyadh 11451, Saudi Arabia

*Khalid A. Al-Rashood*, College of Pharmacy, King Saud University,  
Riyadh 11451, Saudi Arabia

*Khalid A. M. Al-Rashood*, College of Applied Medical Sciences, King  
Saud University, Riyadh 11451, Saudi Arabia

*Fahad J. Al-Shammary*, College of Applied Medical Sciences, King Saud  
University, Riyadh 11451, Saudi Arabia

*Syed Laik Ali*, Zentrallaboratorium Deutscher Apotheker, 6236 Eschborn,  
Federal Republic of Germany

*Said M. Bayomi*, College of Pharmacy, King Saud University, Riyadh  
11451, Saudi Arabia

*Ingrid Becue*, Ciba-Geigy Corporation, Suffern, New York 10901

*Gerald A. Brenner*, Merck Sharp & Dohme Research Laboratories, West  
Point, Pennsylvania 19486

*Glenn A. Brewer*, The Squibb Institute for Medical Research, New Brun-  
swick, New Jersey 08903

*Harry G. Brittain*, The Squibb Institute for Medical Research, New  
Brunswick, New Jersey 08903

*Auke Bult*, Faculty of Pharmacy, State University of Utrecht, 3511 GH  
Utrecht, The Netherlands



- James E. Carter*, Janssen Pharmaceutical, Piscataway, New Jersey 08854
- Seham S. El-Hawary*, College of Pharmacy, King Saud University, Riyadh 11451, Saudi Arabia
- Humeida A. El-Obeid*, College of Pharmacy, King Saud University, Riyadh 11451, Saudi Arabia
- Klaus Florey*, The Squibb Institute for Medical Research, New Brunswick, New Jersey 08903
- George A. Forcier*, Pfizer Inc., Groton, Connecticut 06340
- Lee T. Grady*, The United States Pharmacopeia, Rockville, Maryland 20852
- Joost J. M. Holthuis*, Faculty of Pharmacy, University of Utrecht, 3511 GH Utrecht, The Netherlands
- Eugene L. Inman*, Lilly Research Laboratories, Indianapolis, Indiana 46285
- Vijay K. Kapoor*, Department of Pharmaceutical Sciences, Panjab University, Chandigarh 160014, India
- J. Jantina Kettenes-van den Bosch*, Faculty of Pharmacy, University of Utrecht, 3511 GH Utrecht, The Netherlands
- Leonard J. Kostek*, Pfizer Incorporated, Central Research, Groton, Connecticut 06340
- G. William Martin*, Burroughs Wellcome Co., Research Triangle Park, North Carolina 27709
- Mohammad Saleem Mian*, College of Pharmacy, King Saud University, Riyadh 11451, Saudi Arabia
- Neelofur Abdul Aziz Mian*, College of Applied Medical Sciences, King Saud University, Riyadh 11451, Saudi Arabia
- Farid J. Muhtadi*, College of Pharmacy, King Saud University, Riyadh 11451, Saudi Arabia
- Gandharva Padmanabhan*, Ciba-Geigy Corporation, Suffern, New York 10901
- Mohammad Riaz*, University of Michigan, Ann Arbor, Michigan 48109
- James B. Smith*, Ciba-Geigy Corporation, Suffern, New York 10901
- Dorothy K. Wyatt*, The United States Pharmacopeia, Rockville, Maryland 20852
- John E. Zaremba*, W. H. Rorer Inc., Fort Washington, Pennsylvania 19034
- Muhammad Uppal Zubair*, Center for University Woman Studies, King Saud University, Riyadh 11451, Saudi Arabia

## PREFACE

Although the official compendia define a drug substance as to identity, purity, strength, and quality, they normally do not provide other physical or chemical data, nor do they list methods of synthesis or pathways of physical or biological degradation and metabolism. Such information is scattered through the scientific literature and the files of pharmaceutical laboratories.

I perceived a need to supplement the official compendial standards of drug substances with a comprehensive review of such information, and seventeen years ago, the first volume of *Analytical Profiles of Drug Substances* was published. That we have been able to publish one volume per year is a tribute to the diligence of the editors to solicit articles and even more so to the enthusiastic response of our authors, an international group associated with pharmaceutical firms, academic institutions, and compendial authorities. I would like to express my sincere gratitude to them for making this venture possible.

Over the years, we have had queries concerning our publication policy. Our goal is to cover all drug substances of medial value, and therefore, we have welcomed any articles of interest to an individual contributor. We also have endeavored to solicit profiles of the most useful and used medicines, but many in this category still need to be profiled.

Klaus Florey

*ANALYTICAL PROFILE OF AZINTAMIDE*

*Ezzat M. Abdel-Moety  
and  
Hamad A. Al-Khamees*

*Department of Pharmaceutical Chemistry,  
College of Pharmacy, King Saud University,  
P.O. Box 2457, Riyadh-11451,  
Saudi Arabia.*

CONTENTS

1. INTRODUCTORY
  2. DESCRIPTION
    - 2-1. Name
    - 2-2. Formulae
    - 2-3. The Chemical Abstract Registry (CAS) Number
    - 2-4. Appearance, Color, Odor and Taste
    - 2-5. Physical Characteristics
    - 2-6. Crystal Characteristics
    - 2-7. Spectral Characterization
  3. SYNTHESIS
  4. PHARMACOLOGY
  5. THERAPEUTIC CATEGORATION AND USES
    - 5-1. Categoration
    - 5-2. Uses
  6. TOXICOLOGY
  7. STABILITY AND DEGRADATION
  8. PHARMACOKINETICS
    - 8-1. Biotransformation
    - 8-2. Absorption
    - 8-3. Excretion
  9. METHODS OF ANALYSIS
    - 9-1. Qualitative Methods
    - 9-2. Quantitative Methods
- ACKNOWLEDGEMENT
- REFERENCES

## 1. INTRODUCTORY

Azintamide is a true potent choleretic drug, which is totally synthesized in 1959. The drug has the registered trade name Oragallin®. In spite of the potent choleretic activity, with moderate cholepoietic action, and its wide therapeutic applications in different conditions and countries, no detailed informations about its physical, chemical, clinical, and bioavailability characteristics have been yet collectively summarized in simple presentation. The present Analytical Profile is an effort in this direction.

## 2. DESCRIPTION

### 2-1. Names

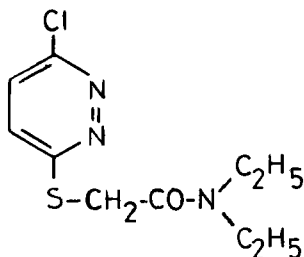
2.11. Chemical: 2-[(6-Chloro-3-pyridazinyl)thio]-N,N-diethylacetamide.

Other chemical names are, N,N-diethyl-2-[6-(3-chloropyridazinyl)thio]acetamide; N,N-diethyl-2-[6-(3-chloropyridazinyl)-mercapto]acetamide; and (3-chloro-6-pyridazinylthio)acetic acid diethylamide (1).

2.12. Proprietary: Oragallin, Ora-gallin, and ST 9067.

Azintamide has been registered under the trade name Oragallin® for Österreichische Stickstoffwerke, AG, Linz/Donau - Austria.

### 2-2. Formula and Molecular Weight



[C<sub>10</sub>H<sub>14</sub>ClN<sub>3</sub>OS (259.77)]

2-3. The Chemical Abstract Registry (CAS) Number:  
[1830-32-6].

2-4. Appearance, Color, Odor, and Taste

Microcrystalline, white, odorless powder with bitter taste.

2-5. Physical Characteristics

2-51. Melting Range

The melting of azintamide was carried out at a heating rate of  $1^{\circ}\text{C}.\text{min}^{-1}$  on a Kofler hot-stage microscope.

Table 1: Melting point and range of azintamide\*

Start temperature ( $^{\circ}\text{C}$ )	Melting range ( $^{\circ}\text{C}$ )	Mid-point ( $^{\circ}\text{C}$ )	Literature ( $^{\circ}\text{C}$ )
90	95.0-97.0 (2.0)	96.0	98-100 (2)
	95.5-97.5 (2.0)	96.5	97-98 (1,3)

\*Sample from Österreichische Stickstoffwerke, AG, Linz/Donau-Austria, BN: 23540/524699 - all values ( $^{\circ}\text{C}$ ) are uncorrected.

2-52. Differential Thermal Scanning (DSC)

The DSC-curve was obtained on a DuPont TA-9900 Thermal Analyzer attached to a data processing unit. Figure 1 shows the DSC-curve of azintamide. The running was between  $50-150^{\circ}\text{C}$  at heating rate of  $10^{\circ}\text{C}.\text{min}^{-1}$ . The heat of activation and the purity of the sample was determined using purity program.

2-53. Solubility

Azintamide is freely soluble in benzene, chloroform, ethyl acetate and acetone, its solubility in water is  $5 \text{ mg}.\text{ml}^{-1}$  (1).

Sample : AZINTAMIDE-B

Size : 4.60 mg

Method : DSC 50 TO 150 10 C/M.

Comment:

DSC

Run Date: 02/11/88 19:13

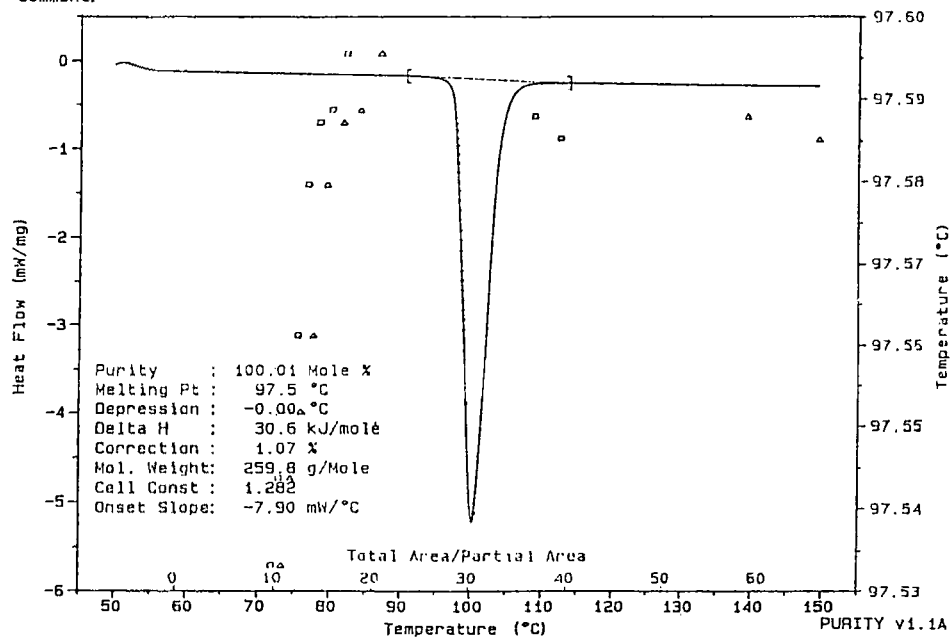


Fig. 1: The differential Scanning Curve (DSC) of Azintamide.

## 2-54. Optical Rotation

Azintamide is an optically-inactive species.

## 2-6. Crystal Characteristics

### 2-61. Crystallization

Azintamide is readily crystallizable from acetone (1).

### 2-62. Crystal Forms

Microscopic examination of the microcrystals of azintamide was carried out by using a Leitz Camera Lucida ( $X = 40$ ) attached to a Leitz projector. Figure 2 shows the different crystal forms of azintamide.

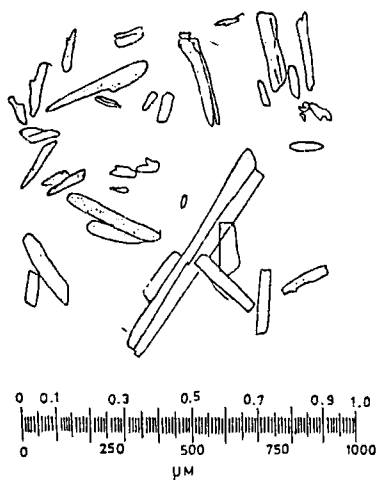


Fig.2: Different Crystal Forms of Azintamide



2-63. X-Ray Powder Diffraction

The X-ray powder diffraction pattern of azintamide was obtained on a Philips' PW1710 Diffractometer with single crystal monochromator and copper K $\alpha$  radiations. The patterns were recorded on a Philips' PM 8210 printing recorder. The values of  $2\theta$ , d-spacing ( $\text{\AA}$ ), and counts were automatically obtained on a Digital printer. Table 2 summarizes the obtained characteristic lines; while figure 3 shows the obtained X-ray diffractometric curve.

Table 2: Characteristic lines of the X-ray diffraction of azintamide powder.

$2\theta$	d( $\text{\AA}$ )	[I/I <sub>0</sub> (%)]	$2\theta$	d( $\text{\AA}$ )	[I/I <sub>0</sub> (%)]
7.179	12.3132	13.14	33.064	2.7092	2.52
7.978	11.0815	52.52	34.213	2.6208	12.79
10.363	8.5357	20.10	35.445	2.5324	2.67
12.029	7.3571	7.85	35.870	2.5034	4.91
12.557	7.0488	6.63	36.454	2.4647	4.18
13.051	6.7832	100.00	36.913	2.4350	2.77
15.925	5.5650	7.48	37.390	2.4051	4.28
16.639	5.3278	4.49	39.936	2.2574	6.63
18.088	4.9041	3.06	40.407	2.2322	2.19
18.704	4.7439	5.13	41.317	2.1851	2.87
19.727	4.5003	45.39	41.985	2.1519	2.91
20.788	4.2724	27.82	42.377	2.1329	15.98
21.532	4.1269	2.90	43.129	2.0974	8.66
21.983	4.0433	7.72	44.420	2.0394	6.11
22.904	3.8827	2.80	45.775	1.9821	2.70
23.995	3.7087	20.02	46.789	1.9415	2.63
24.664	3.6094	7.42	47.036	1.9319	2.08
25.211	3.5324	4.78	48.034	1.8941	3.67
25.889	3.4414	2.83	49.226	1.8510	8.22
26.875	3.3173	11.39	50.482	1.8078	5.36
27.365	3.2591	4.99	52.793	1.7340	2.99
27.779	3.2114	2.09	56.196	1.6368	4.13
28.868	3.0927	10.18	56.572	1.6268	2.16
30.053	2.9734	3.07	58.426	1.5795	5.00
30.711	2.9112	44.95	60.288	1.5351	5.06
32.075	2.7904	30.49			

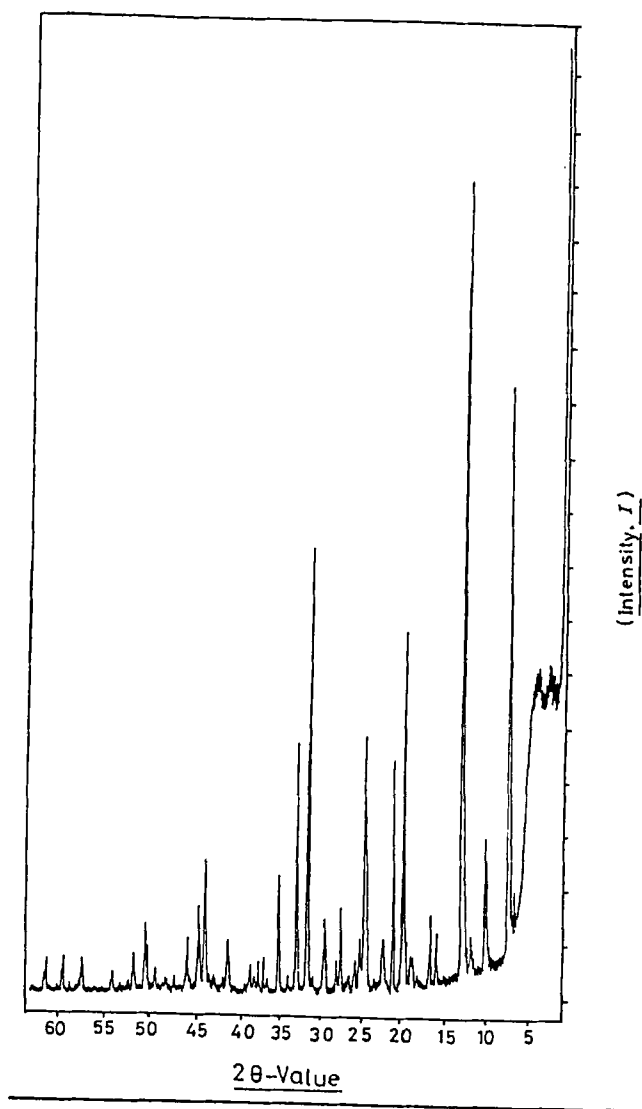


Fig. 3: The X-Ray Powder Diffraction of Azintamide.

## 2-7. Spectral Characterization

### 2-71. Ultraviolet (UV) Spectrum

The UV-scanning of  $5 \mu\text{g} \cdot \text{ml}^{-1}$  solutions of azintamide solutions in water, 0.1-N HCl, and 0.1-N NaOH is given in figure 4. The spectral running was carried out on a DMS 90 Varian double-beam UV/visible spectrophotometer attached to a Hewlett-Packard 7015 B X-Y chart recorder and using 1-cm quartz cells. Table 3 presents collectively the obtained  $A(1\%, 1 \text{ cm})$  values, molar absorptivities, and the ratios of absorbances ( $A$ -ratios) at about 316 nm, 306 nm, and 258 nm of azintamide solution in 95% ethanol.

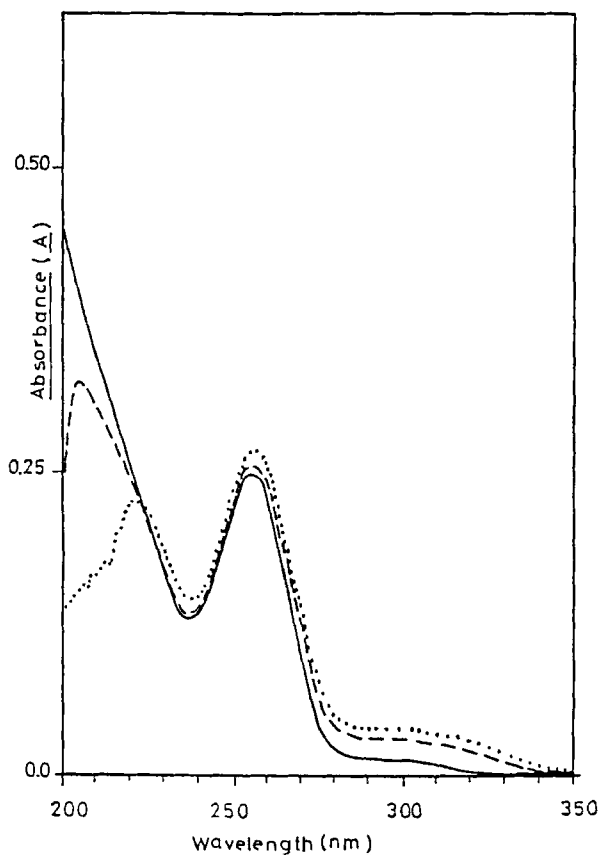


Fig. 4: The UV-scanning of  $5 \mu\text{g} \cdot \text{ml}^{-1}$  Solutions of Azintamide (—), 0.1N HCl (---), and 0.1N NaOH(....)

Table 3: The ultraviolet absorption of azintamide (in ethanol)

Wavelength* (nm)	A(1%, 1 cm)	$\epsilon$
316	54.08	$1.404 \times 10^3$
306	57.43	$1.492 \times 10^3$
258	547.00	$1.421 \times 10^4$

\* A-ratios =  $A_{258/316} = 10.114$  and  $A_{258/306} = 9.525$

## 2-72. Infrared (IR) Spectrum

The IR spectrum was carried out on a Perkin-Elmer 1310 IR-Spectrometer, in KBr (ca. 1%). Figure 5 shows the IR spectrum of azintamide, and table 4 collects the IR band assignments of the drug.

Table 4: The infrared band assignments for azintamide

Wave No. (cm <sup>-1</sup> )*	Assignment
2980 (s), 2945 (m)	CH-stretching in heteroaromatic ring
1630-1636 (s)	C = O stretching, amide
1580 (m)	N = N stretching
1500-1420 (m)	CH <sub>2</sub> -scissoring with CO and S
1410 (s)	C-N stretching, amide
860 (m)	C-Cl and C-N stretching

\* m = medium, s = strong

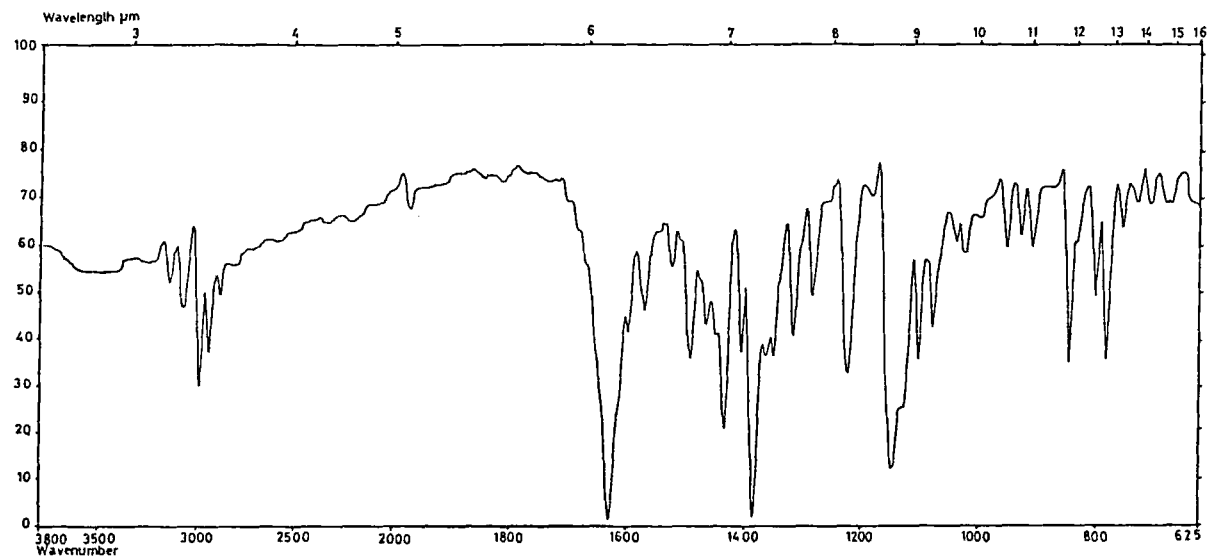
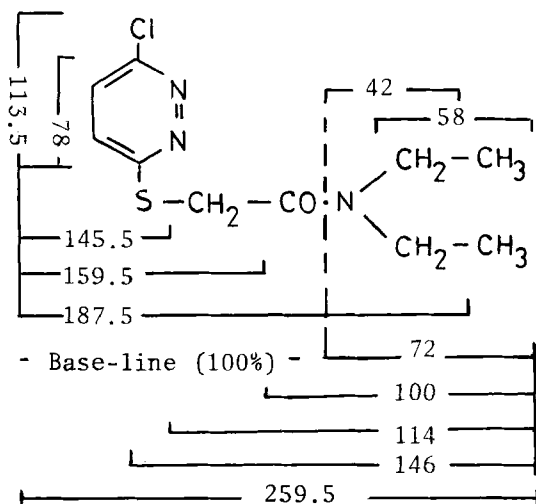


Fig. 5: The Infrared (IR) Spectrum of Azintamide (~ 1%, KBr).

### 2-73. Mass Spectrum

The low resolution mass spectrum of azintamide is presented in figure 6. The running was made on a Varian CH-7 Mass Spectrometer. Table 5 shows the possible mass fragmentations obtained from mass spectrometric measurement of the drug by introducing it directly using shove pole.

The following scheme shows the possible mass fragmentation pattern of azintamide



Azintamide

### 2-74. Nuclear Magnetic Resonance (NMR) Spectra

The proton as well as the  $^{13}\text{C}$  carbon nuclear magnetic resonances of azintamide were carried out on the drug solution in  $\text{CDCl}_3$  by using TMS as internal standard on a Varian XL-200 spectrometer.

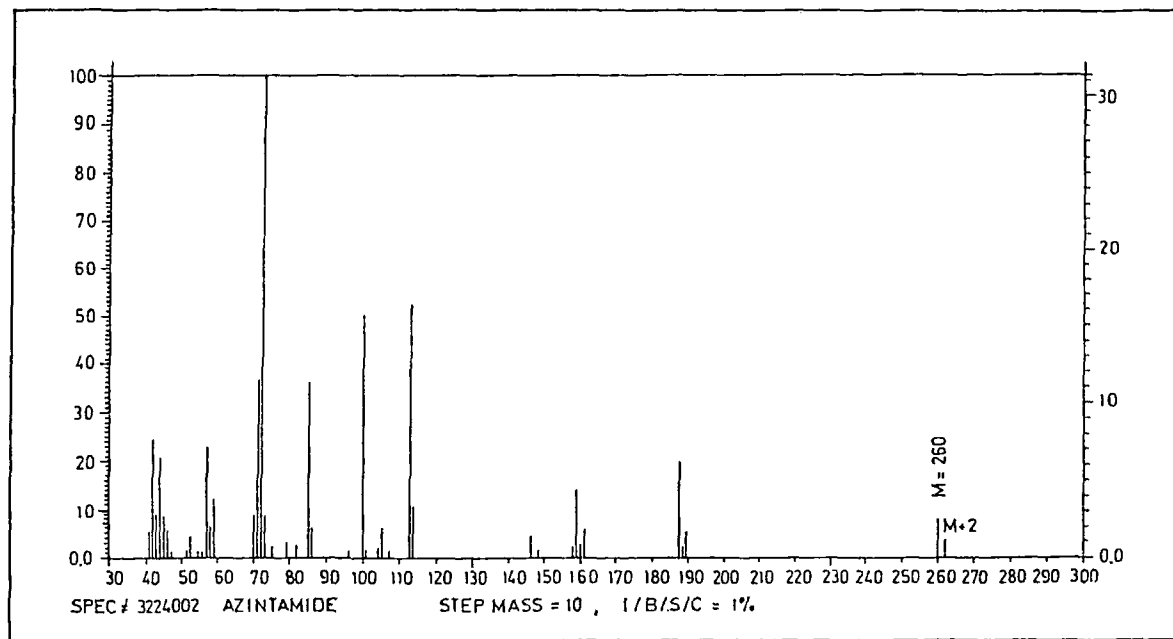
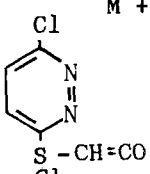
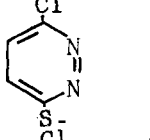
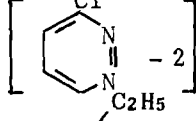
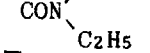
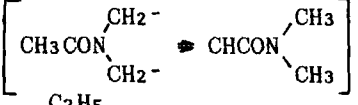
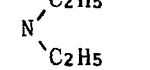
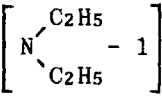
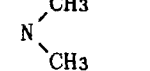
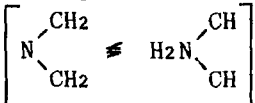


Fig. 6: The 70 eV Mass Spectrum of Azintamide.

Table 5: Low resolution mass spectrometric assignments of azintamide

Measured mass*	Structural Assignment	Formula
262 (4)	M + 2	C <sub>10</sub> H <sub>16</sub> ClN <sub>3</sub> OS
187 (19)		C <sub>6</sub> H <sub>3</sub> ClN <sub>2</sub> OS
159 (15)		C <sub>5</sub> H <sub>3</sub> ClN <sub>2</sub> S
113 (50)		C <sub>4</sub> HClN <sub>2</sub>
100 (50)		C <sub>5</sub> H <sub>6</sub> NO
85 (46)		C <sub>4</sub> H <sub>7</sub> NO
72 (100) base-peak		C <sub>4</sub> H <sub>6</sub> N
71 (37)		C <sub>4</sub> H <sub>5</sub> N
58 (12)	CH <sub>2</sub> -CH <sub>3</sub>	C <sub>2</sub> H <sub>5</sub>
57 (8)	CH <sub>3</sub> CON $\rightleftharpoons$ CHCONH <sub>2</sub>	C <sub>2</sub> H <sub>3</sub> NO
56 (24)	CH <sub>2</sub> CON $\rightleftharpoons$ CCONH <sub>2</sub>	C <sub>2</sub> H <sub>2</sub> NO
44 (22)		C <sub>2</sub> H <sub>6</sub> N
42 (25)		C <sub>2</sub> H <sub>4</sub> N

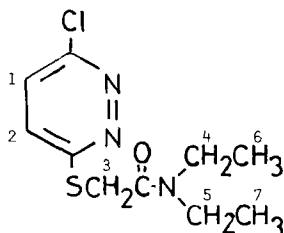
\* Figures in parenthesis are the percent relative intensity of the peak.



2-741. Proton Nuclear Magnetic Resonance ( $^1\text{H-NMR}$ ) Spectrum

The 200 MHz  $^1\text{H-NMR}$  spectrum of azintamide is shown in figure 7. The spectral peak assignments of the drug are presented in Table 6.

Table 6: The  $^1\text{H-NMR}$  spectral assignments for azintamide



Chemical shift ( $\delta$ , ppm)	Proton assignments ( $\text{CDCl}_3$ )*
7.46	[d, 1H (1 or 2), aromatic]
7.37	[d, 1H (1 or 2), aromatic]
4.33	[s, 2H (3), $\text{SCH}_2\text{CO}$ ]
3.52-3.41	[m, 4H (4 & 5), $2\text{CH}_2\cdot\text{CH}_3$ ]
1.32-1.25	[t, 3H (6), $\text{CH}_2\text{CH}_3$ ] <sup>+</sup>
1.18-1.11	[t, 3H (7), $\text{CH}_2\text{CH}_3$ ] <sup>+</sup>

\* s, d, t, m, are symbols for singlet, doublet, triplet, and multiplet, in order; figure in parentheses are the location numbers.

<sup>+</sup> The non-equivalence of the two methyl groups of the aliphatic amide in azintamide is due to restricted steric

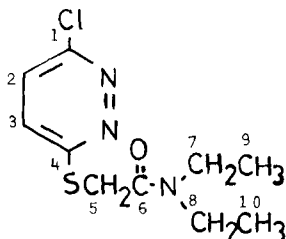
rotation about  $\text{C} \begin{array}{c} \text{O} \\ \parallel \\ \text{N} \end{array} \text{N}$  (5).



2-742.  $^{13}\text{C}$ Carbon Nuclear Magnetic Resonance Spectrum ( $^{13}\text{C}$ -NMR)

The 200 MHz  $^{13}\text{C}$ -NMR spectrum of azintamide is shown in Figure 8. The spectral peak assignments of the drug are presented in Table 7.

Table 7: The  $^{13}\text{C}$ -NMR spectral assignments for azintamide



Chemical shift ( $\delta$ , ppm)	Carbon assignment ( $\text{CDCl}_3$ )*
12.85	[1C (10), C. <u>C</u> H <sub>3</sub> ] <sup>†</sup>
14.28	[1C (9), C. <u>C</u> H <sub>3</sub> ] <sup>†</sup>
34.10	[1C (5), S <u>C</u> H <sub>2</sub> CO]
40.76	[1C (7), N. <u>C</u> H <sub>2</sub> .C] <sup>†</sup>
42.52	[1C (8), N. <u>C</u> H <sub>2</sub> .C] <sup>†</sup>
127.39	[1C (2), CH-aromatic]
128.16	[1C (3), CH-aromatic]
153.60	[1C (1), C-aromatic)]
161.21	[1C (4), C-aromatic]
166.01	[1C (6), C. <u>C</u> O.N ]

\* Figure in parentheses are the location numbers.

<sup>†</sup> Non-equivalence due to restricted steric rotation about the aliphatic amide (5).

Fig. 8: The 200-MHz  $^{13}\text{C}$ -NMR Spectrum of Azintamide.

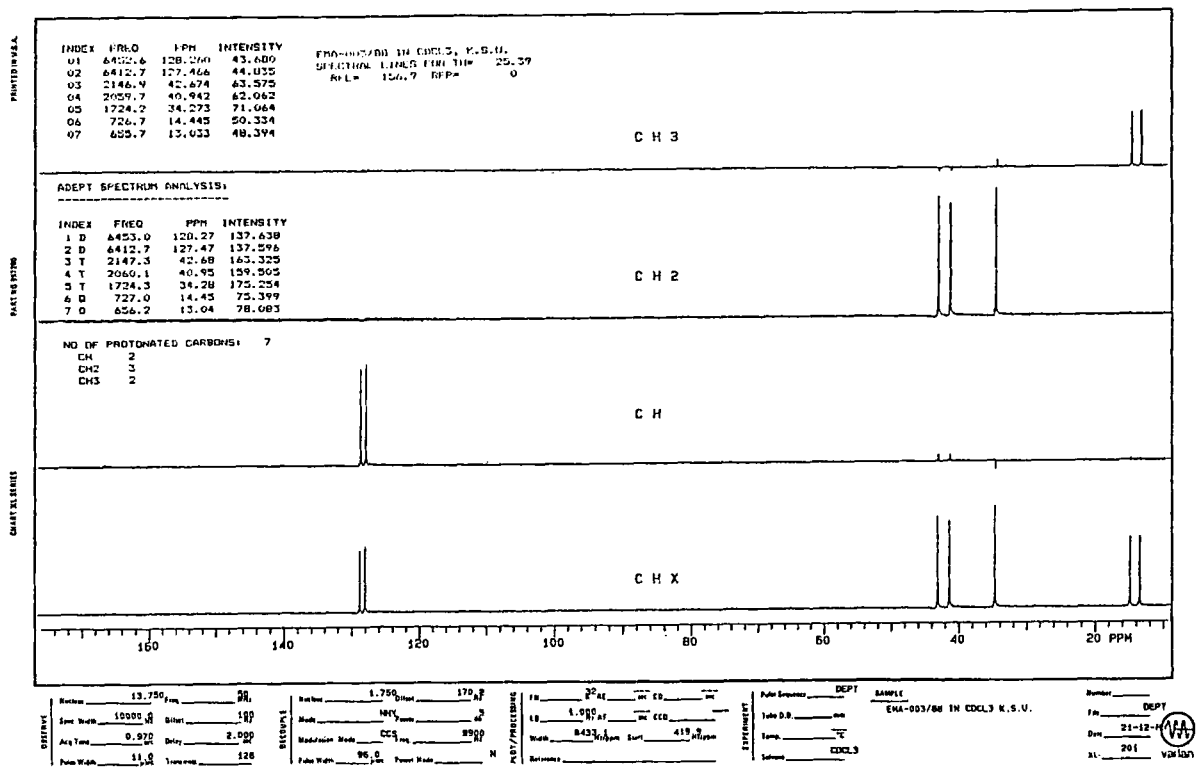


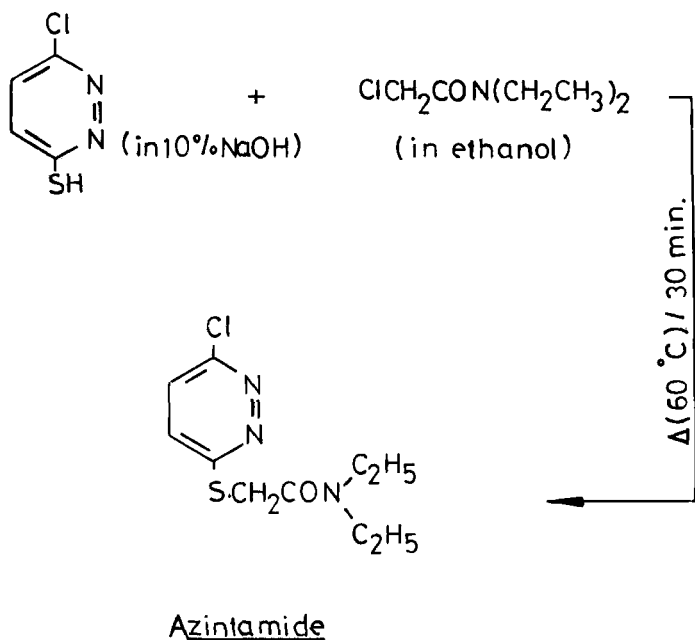
Fig. 9: The 200-MHz DEPT Spectrum of Azintamide.

The Distortionless Enhancement by Polarization Transfer (DEPT) at 200 MHz for azintamide was run on the same sample of the drug in  $\text{CDCl}_3$  against TMS as internal standard; figure 9 shows the typical classification of protonated carbons ( $\text{CHX}$ ) to  $\text{CH-}$ ,  $\text{CH}_2-$  and  $\text{CH}_3-$  carbons.

### 3. SYNTHESIS

Azintamide has been synthesized in 1959 by Schönbeck (6) among various pyridazine derivatives having different choleric activities (3). Azintamide (ST 9067) was assigned and patented (German Patents: 1188604, Nov. 1965) to Lentia GmbH, Munich-F.R. Germany (7).

The following scheme illustrates the synthetic pathway of azintamide:



Azintamide, [3-chloropyridazinyl-6-thio]-acetic acid diethylamide, could be obtained by reacting 7.3 parts of 3-chloro-6-methylthiopyridazine (8) dissolved in 20 parts of 10% NaOH with a solution of 7.5 parts chloroacetic acid diethylamide in 20 parts alcohol at 60°C for 30 min. Crystallization can occur by cooling to 0°C and recrystallization from acetone (3).

#### 4. PHARMACOLOGY

The choleric activity of azintamide has been predicted firstly by comparing with those of the 2-[(6-chloro-3-pyridazinyl)thio] acetic acid (ST 9024), synthesized by Schönbeck (6). The substituted amide showed the highest potency (3,6). The choleric activity of azintamide has been carried out on rats against dehydrocholic acid as reference substance (9), then on human volunteers (10-13). Choleresis due to azintamide in man with rapid onset was manifested within a short administration time. Azintamide is now one of the most recommended choleric references for naturally occurring plant cholagogues (14) and other synthetic cholericly active compounds (15). Azintamide, in identical doses, evokes a more powerful choleresis than either of dehydrocholic acid and 1-phenylpropanol (9). The elimination of bromosulfo-phthalene (BSP) is only influenced in the first 30-minutes period following intraduodenal administration of 50 mg.Kg<sup>-1</sup> dose. The same dose of dehydrocholic acid, in every cases, retarded BSP-elimination over all 30 minutes periods. The choleric activity of the drug can be demonstrated in selected patients by means of Bartelheimer's double ballon tube. An increase in bile flow, depending on the dose, was stated after administration of 1 g azintamide. This demonstrates the drug has true choleric increasing secretion of the components of the bile and not merely increasing fluid volume, i.e. hydrocholeresis. No increase of serum bilirubin was observed after azintamide and the maximum effect is reached within 20 to 40 minutes after administration.

## 5. THERAPEUTIC CATEGORATION AND USES

### 5-1. Categoration

Azintamide is a potent choleretic drug substance which exerts also a moderate cholepoietic action as well. The drug can be categorized as antihypercholesterolemic substance because it can reduce the serum cholestrol, non-esterified fatty acid and free glycerol; i.e. the drug can be considered as antihyperlipidemic.

### 5-2. Uses

Fatty indigestion, cholangitis, cholecystitis, icteric and posticteric cases, liver protection in conditions of cholecystopathy, meteorism and hepatogenic dermatosis, such as *psoriasis vulgaris* (16). The drug can be used in some cases of hypercholesterolemia (17,18). Azintamide can be prescribed for hepatitis after treatment and for Roemheld-syndromes.

### 5-21. Contraindications

As all choleretics, azintamide should be avoided in cases of acute hepatitis and in cases inclining to biliary colic due to cholelithiasis, as choleresis can cause mobilization of gall stones, possibly resulting in partial or complete occlusion of the bile duct.

### 5-22. Dosage

150-300 mg.day<sup>-1</sup> is the normal adult dose, which can be taken during meal once or divided to three times.

## 6. TOXICOLOGY

Acute Toxicity (oral by mice): A-LD<sub>50</sub> is 2.34 g.Kg<sup>-1</sup> (1.94-2.48) (9, 10).

Chronic Toxicity (6 months term): C-LD<sub>50</sub> is 1.18 g.Kg<sup>-1</sup> (0.97-1.43) (19).



## 7. STABILITY AND DEGRADATION

Lindner *et al.* (19) demonstrated that azintamide decomposes due to the hydrolysis of the amide linkage with formation of diethylamine and [3-chloropyridazinyl-6-thio] acetic acid as the principal degradation products.

## 8. PHARMACOKINETICS

### 8-1. Biotransformation

Through amide-linkage break, [3-chloropyridazinyl-6-thio]acetic acid is identified in rats' urine as the main metabolic product (19). Only small amount of the unchanged drug could be traced in plasma and urine. The principal metabolites, i.e. [3-chloropyridazinyl-6-thio]acetic acid and diethylamine, are subject for further biotransformation such as s-oxidation or detoxification as sulfate and or glucuronate conjugates.

### 8-2. Absorption

The main part of the intaken azintamide is absorbed and reabsorbed from intestine (19). Sometimes, azintamide may be dispensed with some digesting enzymes, e.g. Wilzym 600, a standardized pancreatic ferments), in such cases the preparation has to be in enteric-coated form to protect the enzyme components from inactivation by stomach acids. The inactivation of these enzymes can also be caused by excess antacids, such as silicates, magnesia, and bicarbonate, or by absorbents like charcoal powder (20).

### 8-3. Excretion

Azintamide is excreted directly or indirectly in the bile (19). Traces of unchanged drug can be investigated in feaces, but the main excretory product is the [3-chloropyridazinyl-6-thio] acetic acid, small amounts in free form and the majority in conjugated bindings.

9. METHODS OF ANALYSIS9-1. Qualitative Methods9-11. Elemental Composition

<u>Element</u>	<u>%-Composition</u>
C	46.24
H	5.43
Cl	13.65
N	16.17
O	6.16
S	12.35

9-12. Identification with Microchemical Tests

Table 8 summarizes the chemical reactions with some common reagents used for identification of azintamide. The color reactions can be also useful for identification of the drug after its chromatographic, paper (PC) and thin-layer (TLC), separation.

Table 8: Microchemical tests for identification of azintamide

<u>Reagent</u>	<u>Observation</u>
* 2% anisaldehyde/conc.H <sub>3</sub> PO <sub>4</sub> + CH <sub>3</sub> COOH + C <sub>2</sub> H <sub>5</sub> OH (3:1:1, v/v/v); heat.	Violet-red color (19)
* 0.5% p-dimethylaminobenzaldehyde (PDAB) HCl + C <sub>2</sub> H <sub>5</sub> OH; heat.	Green-blue color (9,19)
* 0.5% PDAB/70% H <sub>2</sub> SO <sub>4</sub>	Yellow-red (21).
* Vanilin/H <sup>+</sup> ; heat	Blue-violet color to turbidity (19).
*5% Silicotangestic acid/HCl	White precipitate, dissolves on boiling.

9-13. Chromatographic Methods9-131. Paper Chromatography (PC)

Table 9 shows the PC-separation and identification of azintamide.

Table 9: The paper chromatography of azintamide

Mobile phase	hRf*	Visualization	Reference
30% acetic acid	88	0.5% PDAB in HCl + C <sub>2</sub> H <sub>5</sub> OH; heat at 70°C/5 min., or under UV-light; green-blue spots appear	(9)

\*hRf is the travelling rate (Rf) X 100.

9-132. Thin-layer Chromatography (TLC)

Azintamide can be chromatographed on different thin-layers either by adopting the one- or the two-dimensional techniques. Table 10 summarizes the TLC-separation and characterization of azintamide.

Table 10: The thin-layer chromatography of azintamide

Mobile phase	Layer	hRf	Visualization	Reference
<u>1. One-dimensional TLC</u>				
*CHCl <sub>3</sub> + C <sub>2</sub> H <sub>5</sub> OH (100:5, v/v)	Silica gel GF254	55	a. UV-light (250 nm) b. Spray with KMnO <sub>4</sub> /OH <sup>-</sup> to give yellow spots on violet background.	(2)
*CH <sub>3</sub> OH + conc. NH <sub>4</sub> OH (100:1.5, v/v) (22)	Silica gel GF254	65-66	a. UV-light (254 nm) b. 2% PDAB in 5% H <sub>2</sub> SO <sub>4</sub> ; gives yellowish green spots after ca. 30 min.	(21)
<u>2. Two-dimensional TLC</u>				
*P <sub>1</sub> : CH <sub>3</sub> COOC <sub>2</sub> H <sub>5</sub> + CH <sub>3</sub> OH + 3-N(NH <sub>4</sub> ) <sub>2</sub> CO <sub>3</sub> + DMF (12:2:1:1, v/v/v/v).	Silica gel G	74	a. 2% anisaldehyde in conc. H <sub>3</sub> PO <sub>4</sub> + CH <sub>3</sub> COOH + C <sub>2</sub> H <sub>5</sub> OH (3:1:1, v/v/v); heat 120°C/ 20 min., to give violet red spots on white to light rose background.	
*P <sub>2</sub> : CCl <sub>4</sub> + CH <sub>3</sub> OH + CH <sub>3</sub> COOH (77.5:20:2.5, v/v/v)		57	b. PDAB/H <sup>+</sup> ; heat 120°C/20 min, to give green blue spots.  c. Vanilin/H <sup>+</sup> ; heat, to give blue violet spots.	

## 9-2. Quantitative Methods

### 9-21. Volumetry

#### 9.211. Titrimetric determination of sulfur and chlorine contents

Azintamide can be assayed *via* its sulfur content after decomposition in Schöniger combustion flask and titration with 0.02-N  $\text{Ba}(\text{ClO}_4)_2$  and thiorine as indicator, taking 12.34 as the theoretical percent S-contents. Determination of the Cl-contents, also after combustion, through titration with 0.01-N  $\text{Hg}(\text{ClO}_4)_2$  and diphenylcarbazone as indicator can be adopted for the drug assay. The theoretical percent content of sulfur is 13.65 (2).

### 9-22. Instrumental Methods

#### 9-221. Colorimetry and spectrophotometry

##### i- Colorimetry

On heating azintamide solution with a solution of p-dimethylaminobenzaldehyde in 70% sulfuric acid, a yellow color is developed with an absorption maximum at 450 nm. Abdel-Moety *et al.* (21) have adopted the color measurement for quantification of the drug in bulk form and in tablets and effervescent granules containing the drug.

##### ii- Direct UV-measurement

The measurement of the light absorption of the drug solution in ethanol at 258 nm is recommended by Abdel-Moety *et al.* (21) for quantitative determination of the drug in raw materials and only in tablets. Recoveries of  $100.16 \pm 1.10$  (n = 6),  $99.84 \pm 1.00$  % (n = 6), in case of tablets, and  $100.30 \pm 1.40$  % (n = 9), for effervescent granules, could be obtained for added azintamide.

##### iii- Derivative spectrophotometry

Abdel-Moety *et al.* (23) described a first-derivative ( $D_1$ ) spectrophotometric procedure for quantification of azintamide in

admixtures with papaverine hydrochloride, a smooth muscle relaxant, which is commonly dispensed with the choleretic drug together to inhibit its possible spasmomimetic activity in the gastrointestinal tract. Amounts of azintamide ( $2-20 \mu\text{g.ml}^{-1}$ , i.e. ppm) and papaverine hydrochloride ( $0.5-6 \mu\text{g.ml}^{-1}$ ) can be accurately quantified. The concentration ranges of both drugs might allow application of the derivative spectrophotometric method to their determination in biological fluids. The  $D_1(dA/d\lambda)$ -spectrophotometric measurement of azintamide is recommended at 264 nm, while for the other component a worked out simultaneous equation can be applied. The recoveries were  $101.02 \pm 0.91\%$  ( $n = 5$ ) and  $100.48 \pm 1.33\%$  ( $n = 5$ ) for azintamide and papaverine hydrochloride in order.

#### iv- PMR-spectrophotometry

El-Khateeb and Abdel-Moety (24) described the application of proton-magnetic-resonance spectrophotometry for quantitative determination of azintamide in pure forms and in dosage formulations. The method involves comparing the integral of both the multiplet centered at about 1.15 ppm and the sharp singlet at 4.30 ppm of azintamide molecule to that of the sharp singlet signal at about 6.30 ppm of maleic acid which is chosen as internal standard.

#### 9-222. Spectrofluorometry

Abdel-Moety *et al.* (25) discussed the coupling possibility of TLC-separation (on layers of silica gel 60 F254) with spectrofluorimetry (EX : 229 nm and EM : 304) for quantification of azintamide in biological fluids. Amounts of  $0.5-2.5 \text{ ng.ml}^{-1}$  (ppb) of the drug in urine samples of a healthy volunteer could be accurately traced. Recovery mean percent of  $97.25 \pm 1.49$  ( $n = 5$ ) could be obtained.

#### 9-223. Flow-injection analysis (FIA)

A single-manifold FIA-system for quantitative determination of azintamide via spectrophotometric detection at 258 nm is investigated by Abdel-Moety *et al.* (26). The limit of quantification and detection is about  $5 \mu\text{g.ml}^{-1}$  of azintamide

dissolved and/or extracted in ethanol could be accurately analyzed. A good percent mean recovery of  $99.24 \pm 0.89$  ( $n = 4$ ) could be obtained at an introduction rate of about  $150 \text{ sample.hr}^{-1}$  or even more. The obtained results were comparable with those of the direct UV-measurement at the same  $\lambda_{\text{max}}$ .

#### 9-224. Chromatographic Techniques

##### i- Gas-liquid chromatography (GLC)

GLC-separation and quantification of azintamide in pharmaceutical formulations has been described by Abdel-Moety (27). The GLC-separation of the drug extracts in chloroform was undertaken on  $150 \text{ cm} \times 4 \text{ mm i.d.}$  column packed with 10% silar on Diatomite C-AW, 100-120 mesh, at  $250^\circ\text{C}$  using  $\text{N}_2$  as carrier gas with flame ionization detection at  $300^\circ\text{C}$ , isothermally. The calibration graph was rectilinear for concentrations  $0.5\text{-}3 \text{ gm ml}^{-1}$  of azintamide with recovery of  $98.77 \pm 1.11\%$  ( $n = 4$ ). The results were compared with those of the spectrophotometric method adopted by the author and others (21). A GC-columns,  $150 \text{ cm} \times 4 \text{ mm i.d.}$ , packed with 5-10% silicone oil on Gas-Chrom Q can elegantly resolve azintamide under the previously mentioned GLC-conditions nearly with identical retention time of about 8 minutes as in case of the silar-column, but the reproducibility in case of the first column was quite better.

##### ii- High-Performanc Liquid Chromatography (HPLC)

HPLC-method for assay of azintamide in one-component dosage forms can be achieved on a  $5\text{-}\mu\text{m}$  C18-Novapack column by using mixture of methanol and water (98:2, v/v) isocratically as a mobile phase at ambient temperatures (28). Another HPLC-procedure is described for quantification of azintamide and papaverine.HCl in binary mixtures and dosage formulations containing both drug substances (29). The separation and quantification can be done on a  $5\text{-}\mu\text{m}$  100 RP-18 LiChrosphere column by using a solvent mixture of acetonitrile and water (56:44, v/v) isocratically at ambient temperature.

### 9-23. Biological Assay

Azintamide can be quantified by the measurement of its choleretic activity according to the procedure described by Stormann (9). The biological measurement is done against the activity of a well known choleretic agent, such as dehydrocholic acid and 1-phenylpropanol. The measurement of the elimination times of bromosulfophthalene (BSP) following suitable intradoudenal doses of the drug in the first 30-minutes.

### 9-3. Other Analytical Possibilites

Different spectroscopic and chromatographic behaviours of azintamide and the various analytical possibilities for the drug have been discussed in a recent communication (30).

---

### ACKNOWLEDGEMENT

The authors would like to thank Dr. R.R. Abou-Shaaban for DSC-investigation and Mr. T.A. Butt for typing the manuscript.



REFERENCES

1. The Merck Index, 10th edn., M. Windholz (Editor), Merck & Co., Inc., Rahway-NJ, 1983, p. 132-No. 915.
2. Private communication; Org. of Pharm. Control Lab. of Österreichische Stickstoffwerke, AG, Linz/Donau-Austria, Dec. 1971.
3. E. Kloimstein, R. Schönbeck, and H. Stormann: Arzneim.-Forsch.; 14, 261 (1964).
4. E.M. Abdel-Moety, A.A. Mostafa, and S.A. Ismaiel: Zentralbl. Pharm. Pharmakother. Laboratoriumsdiagn.; 124, 730 (1985).
5. R.M. Silverstein, G.C. Bassler, and T.C. Morrill (Editors), Spectrometric Identification of Organic Compounds, 4th edn, J. Wiley & Sons, New York, 1981, p. 198.
6. R. Schönbeck: Mh. Chem.; 90, 284 (1959); through Reference 3.
7. E. Kloimstein, R. Schönbeck, and H. Stormann: German Patents; 1188604 (1965 to Lentia GmbH, Munich-F.R. Germany).
8. J. Druey, K.D. Meier, and K. Eichenberger: Helv. Chim. Acta; 37, 133 (1954).
9. H. Stormann: Arzneim.-Forsch.; 14, 266 (1964).
10. G. Hitzenberger: Arzneim.-Forsch.; 14, 279 (1964).
11. W. Neugebauer: Landarzt; 41, 33 (1965).
12. H.L. Dierel: Wien-Med-Wochenschr.; 115, 613 (1965).
13. A. Miyoshi, M. Ito, S. Nakanishi, T. Hagiuro, and T. Takeda: Naika-Hokan; 15, 77 (1968).
14. R. Hansel: Dtsch. Apoth. Ztg.; 125, 1373 (1985).
15. G. Hofrichter, H. Hampel, H.D. Liehn, and E. Ludwig: Arzneim.-Forsch.; 24, 111 (1974).

16. D. Konstantinov and L. Stanoeva: Med-Arh; 29, 117 (1975).
17. P. St.-Janiak: Drugs Future; 3, 191 (1978).
18. J.N. Pereira and G.T. Holland: Disorders of Lipid Metabolism; Annual Reports in Medicinal Chemistry, R.V. Heinzelman (Editor), Vol. 10, Academic Press, New York, 1975, p. 182-219.
19. I. Lindner, H. Stormann, and W. Wendtland: Arzneim.-Forsch.; 14, 271 (1964).
20. Private communication; Chemie Linz, AG' Scientific Office in Riyadh - Saudi Arabia, Feb. 1989.
21. E.M. Abdel-Moety, F.H. Wahdan, N.A. Sharaby, and S.A. Ismaiel: Acta Pharm. Jugosl.; 34, 223 (1984).
22. I. Sunshine: Am. J. Clin. Pathol.; 40, 576 (1963).
23. E.M. Abdel-Moety, M.E. Mohamed, M.A. Abounassif, and M. Al-Awady: Sci. Pharm.; in press (1989).
24. S.Z. El-Khateeb and E.M. Abdel-Moety: Anal. Lett.; 208, 1115 (1987).
25. E.M. Abdel-Moety, S.Z. El-Khateeb, and M.A. Abounassif: Under publication.
26. E.M. Abdel-Moety, A.A. Moustafa, A.K.S. Ahmad, and A.E. El-Gendy: Sci. Pharm.; 55, 259 (1987).
27. E.M. Abdel-Moety: J. Chromatogr.; 324, 475 (1985).
28. S.Z. El-Khateeb and E.M. Abdel-Moety: Sci. Pharm.; in press (1989).
29. M.A. Abounassif, E.M. Abdel-Moety, and E.A. Gad-Kariem: Under publication.
30. E.M. Abdel-Moety: Zentralbl. Pharm. Pharmakother. Laboratoriums-diagn.; 127, 137 (1988).

CHLOROTHIAZIDE

Harry G. Brittain

Squibb Institute for Medical Research

1 Squibb Drive

New Brunswick, NJ 08903

## TABLE OF CONTENTS

1. Description
  - 1.1 Name, Formula, and Molecular Weight
  - 1.2 Appearance
  - 1.3 History
2. Synthesis
3. Physical Properties
  - 3.1 Infrared Spectrum
  - 3.2 NMR Spectra
  - 3.3 Mass Spectra
  - 3.4 Crystallographic Properties
  - 3.5 Hygroscopicity
  - 3.6 Optical Activity
  - 3.7 Melting Phenomena
  - 3.8 Differential Scanning Calorimetry
  - 3.9 Thermogravimetry
  - 3.10 Ionization Constant
  - 3.11 Solubility
  - 3.12 Partition Coefficients
  - 3.13 Ultraviolet Spectrum
  - 3.14 Fluorescence Spectrum
4. Methods of Analysis
  - 4.1 Elemental
  - 4.2 Spectrophotometric
  - 4.3 Chromatographic
    - 4.3.1 Thin-Layer
    - 4.3.2 High Performance Liquid
  - 4.4 Electrochemical
5. Stability
  - 5.1 Solid Stability
  - 5.2 Solution Stability
  - 5.3 Incompatibilities
  - 5.4 Stability in Biological Fluids
6. Drug Metabolism, Pharmacokinetics
7. Acknowledgement
8. References

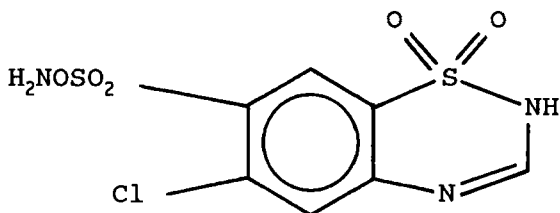
## 1. Description

### 1.1 Name, Formula, and Molecular Weight

The USAN name is chlorothiazide, while the systematic chemical name is 2H-1,2,4-benzothiadiazine-7-sulfonamide, 6-chloro-1,1-dioxide.

The Chemical Abstracts identification number is CAS-58-94-6.

The chemical formula is:



M.W. 295.74

The elemental composition is C 28.43%, H 2.05%, Cl 11.99 %, N 14.21%. O 21.64%, and S 21.69%.

Solid dosage forms are known under the trade names Alurene, Chlorosal, Chlorurit, Chlotride, Clotride, Diuril, Diurilix, Diurite, Flumen, Minzil, Neo-Dema, Salisan, Salunil, Saluretil, Saluric, Warduzide, and Yadalan.

Chlorothiazide for injection is a sterile mixture of chlorothiazide sodium and mannitol, and is known under the trade names Lyovac Diuril, and Sodium Diuril.

### 1.2 Appearance

Chlorothiazide is a white crystalline, odorless powder.

### 1.3 History

Chlorothiazide is a nonmercurial diuretic and antihypertensive, and is a member of the benzothiadiazide class of compounds. These were first synthesized during studies on carbonic anhydrase inhibitors. It was learned that the dominant action of thiazides was to increase renal excretion of sodium and chloride, and an accompanying volume of water. Unlike both the mercurial based agents and carbonic anhydrase inhibitors, the action of thiazides was found to be virtually independent of acid-base balance [1-3].

Chlorothiazide is only one member of the thiazide series, whose functionalities have been systematically examined with respect to diuretic activity. The following generalizations have been proposed: (1) stable substitution on the 7-sulfonamide group destroys carbonic anhydrase activity, (2) various halogen substitutions at the 6 position enhance its diuretic potency, (3) saturation of the heterocyclic ring between the 3 and 4 positions increases potency, (4) certain substituents at the 3 position increase diuretic activity, and (5) addition of various functional groups at the 1, 4, or 5 positions decreases diuretic activity [1-5].

## 2. Synthesis

The synthesis of chlorothiazide has been fully described in the literature [6,7], and was covered by U.S. patents 2,809,194 and 2,937,169 (both to Merck & Co.).

The first step in the synthesis of chlorothiazide involves chlorosulfonation of m-chloroaniline at 150°C (in the presence of sodium chloride) to yield 6-amino-4-chlorobenzene-1,3-disulfonyl chloride, which is treated with ammonium hydroxide to give 6-amino-4-chlorobenzene-1,3-disulfonamide. This compound is then heated with formic acid under reflux to obtain chlorothiazide.

### 3. Physical Properties

#### 3.1 Infrared Spectrum

The infrared absorption spectrum of chlorothiazide was obtained in a KBr disc, and is shown in Figure 1. The principal bands suitable for identification are located at 1157, 1305, and 1342  $\text{cm}^{-1}$ .

#### 3.2 Nuclear Magnetic Resonance Spectrum

The  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra of chlorothiazide have been reported [8], where it was conclusively demonstrated that the compound existed solely as the 4H tautomer in  $\text{DMSO-d}_6$  or acetone- $\text{d}_6$  solution. The resonance position data are summarized in Table I.

#### 3.3 Mass Spectra

The electron impact mass spectrometry of chlorothiazide has been studied during a study involving the MS behavior of nineteen diuretic agents of clinical importance [9]. Chlorothiazide was not found to yield a molecular ion, contrary to a previous report which indicated greater than 50% yield of the molecular ion at  $m/z$  of 295 [10]. The only prominent lines in the MS spectrum above 100 were at  $m/z$  149 (40%), 266 (the base peak), 267 (75%), and 268 (18%). The ions of higher mass were thought to arise through loss of HCN, followed by  $\text{H}^+$  and  $\text{H}_2$  from the molecular ion.

#### 3.4 Crystallographic Properties

The powder x-ray diffraction pattern of chlorothiazide was obtained using a copper K-alpha source (1.54060 Å), and is reproduced in Figure 2. A total of 16 peaks were detected at scattering angles between 2 and 32 degrees 2-theta. The two most diagnostic scattering peaks suitable for identification were observed at 14.6 degrees 2-theta (D-spacing of 6.06 Å), and 29.3 degrees 2-theta (3.04 Å). A full summary of scattering

**Figure 1. Fourier transform infrared spectrum of chlorothiazide, obtained in a KBr disc.**

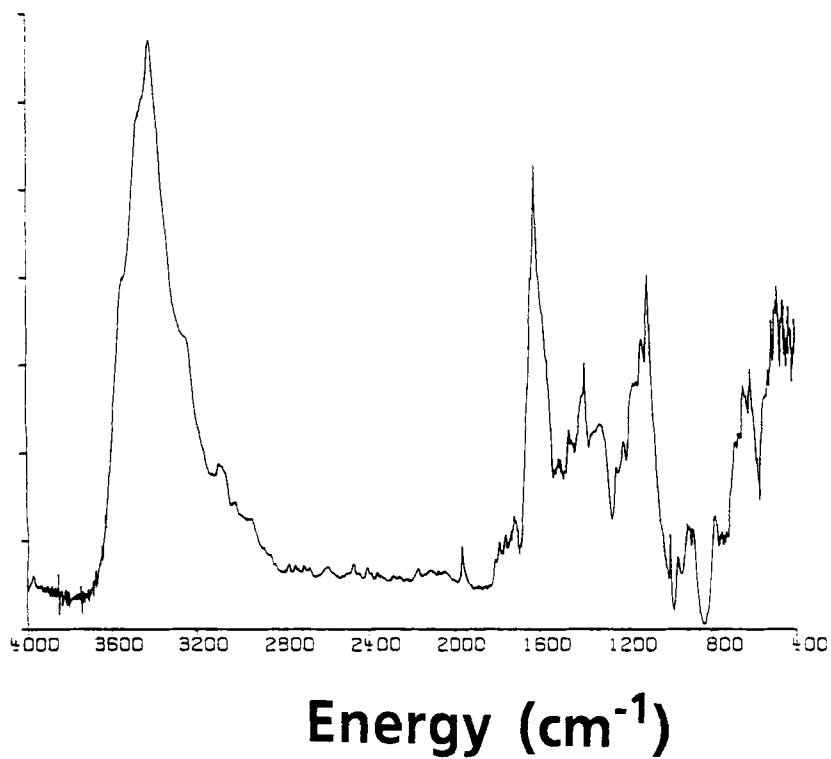




Table I

**<sup>1</sup>H and <sup>13</sup>C Nuclear Magnetic Resonance Resonances  
Observed in DMSO-d<sub>6</sub> Solutions of Chlorothiazide**

<u>Resonance (ppm)</u>	<u>Assignment</u>
(a) <sup>1</sup> H NMR chemical shift values	
8.10	H at C1
8.26 , 7.51	aromatic H
11-13	H of the -NH group
(b) <sup>13</sup> C NMR chemical shift values	
149.7	C3
135.6	C5
121.5	C6
139.0	C7
139.9	C8
126.2	C9
121.4	C10

angles, D-spacings, and relative intensities is found in Table II.

No single crystal structure determination has apparently been reported for chlorothiazide, but a comparison of the data in Figure 2 and Table II with literature data for hydrochlorothiazide [11] indicates the existence of a different space group for chlorothiazide.

### 3.5 Hygroscopicity

As received, chlorothiazide is essentially anhydrous, with typical moisture values around 0.1% being determined using thermogravimetry. The hygroscopicity of chlorothiazide was evaluated by exposing the material to controlled relative humidity environments (over saturated salt solutions), and determining any increase in volatile content. After one week exposure, the moisture uptake was evaluated using thermogravimetry. Even at relative humidity values up to 84%, the total volatile content was found to be less than 1%. From these observations, it is concluded that chlorothiazide is essentially non-hygroscopic.

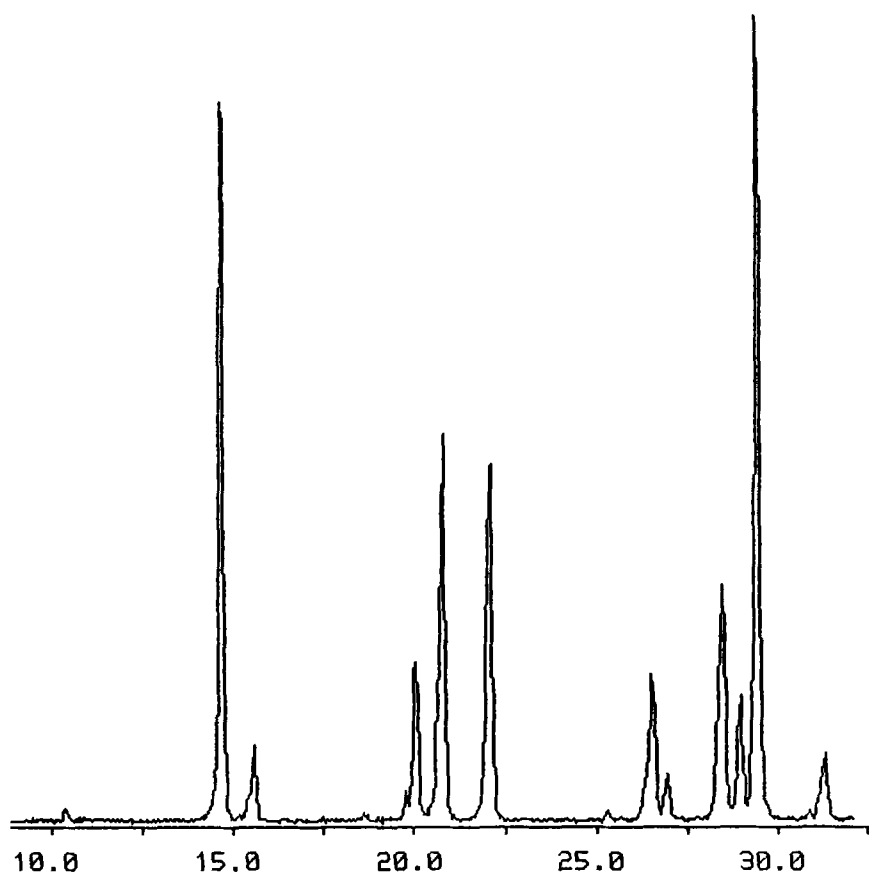
### 3.6 Optical Activity

Chlorothiazide contains no dissymmetric centers, and therefore is not optically active. It can neither exhibit optical rotation or circular dichroism.

### 3.7 Melting Phenomena

When investigated by hot-stage microscopy, chlorothiazide is observed to melt with decomposition beginning around 330°C. Upon close examination, gas bubbles can be detected in the melt, and the melted mixture quickly turns brown as the decomposition proceeds.

**Figure 2. Powder X-ray diffraction pattern of chlorothiazide.**



**Scattering Angle (Degrees 2-θ)**

Table II

**Powder X-ray Diffraction Data Obtained for  
Chlorothiazide: Scattering Angles, D-spacings, and  
Relative Intensities**

Scattering Angle (degrees 2-theta)	D-Spacing Angstroms)	Relative Intensity ( $I/I^{\max}$ )
5.30	16.66	0.48
10.33	8.56	1.59
14.60	6.06	84.63
15.52	5.70	8.67
16.35	5.42	0.29
18.57	4.77	0.95
19.98	4.44	20.29
20.70	4.29	44.57
21.98	4.04	46.58
25.22	3.53	1.06
26.46	3.37	18.85
26.86	3.32	5.13
28.36	3.14	30.95
28.86	3.09	15.27
29.32	3.04	100.00
31.20	2.86	8.04

### 3.8 Differential Scanning Calorimetry

The full differential scanning calorimetric thermogram of chlorothiazide is shown in Figure 3a. No desolvation endotherms were observed at low temperature values, and the only observable DSC features was the melting/decomposition phenomena noted above 330°C. As evident in the expanded DSC thermogram of Figure 3b, the initial melting endotherm exhibits an apparent endotherm at 351°C. The exact temperature associated with the melting process cannot be determined with certainty, since the strong exotherm at 358°C (reflecting oxidative decomposition) undoubtedly cuts off the full endothermic melt.

### 3.9 Thermogravimetry

Thermogravimetric analysis of chlorothiazide generates extremely simple thermograms. Consistent with its analysis as an anhydrous and non-hygroscopic material, no weight loss is observed below 200°C. The first derivative of the TG curve reveals that the initial stages of the decomposition weight loss begin around 355°C, thereby identifying the strong exotherm of the DSC thermogram as an oxidative decomposition feature.

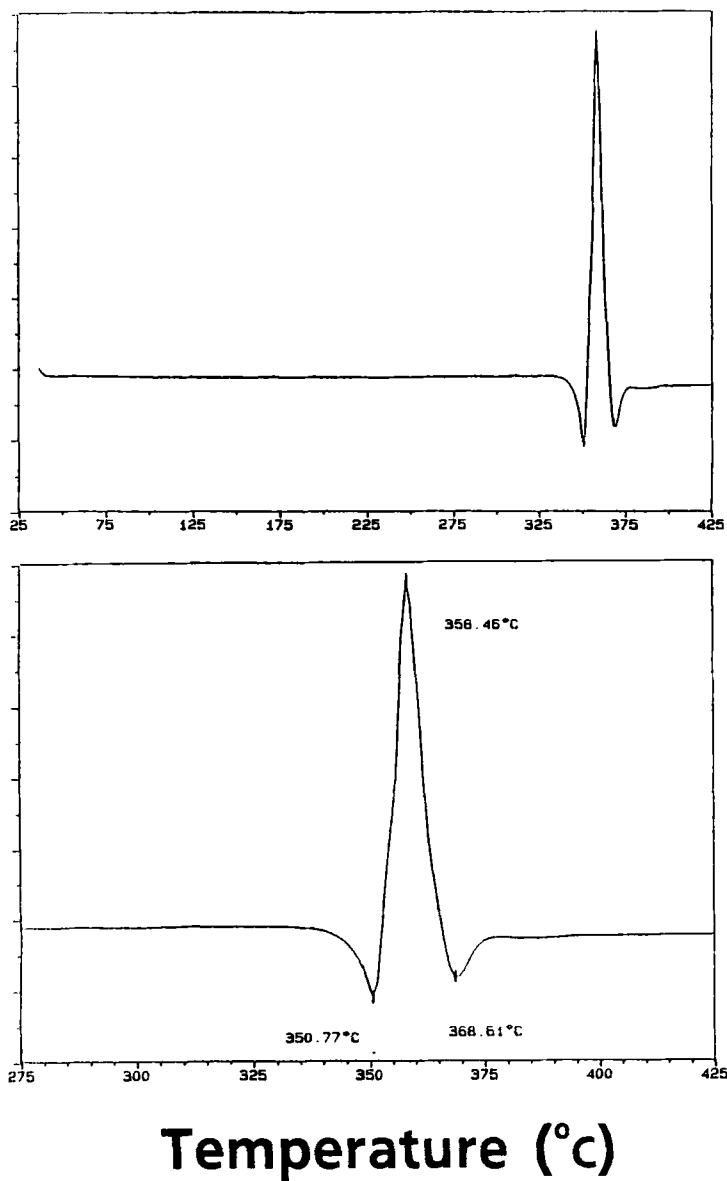
### 3.10 Ionization Constants

The relative insolubility of chlorothiazide in most common solvents has made the determination of ionization constants difficult. Several groups have used aqueous potentiometric titration to obtain  $pK_a$  data, with the general consensus being that  $pK_{a1} = 6.85$  and  $pK_{a2} = 9.45$  [12-14]. Other data have been obtained using UV spectrophotometry, and although no value for  $pK_{a1}$  could be measured, it was found that  $pK_{a2} = 9.7$  [15].

### 3.11 Solubility

The solubility of chlorothiazide in a variety of common solvents has been studied, and it has been noted that the compound is essentially

Figure 3. Full differential scanning calorimetric thermogram of chlorothiazide (upper trace), and expanded view of the decomposition region (lower trace).



insoluble in most solvents. The major exceptions were noted with dimethyl sulfoxide and dimethylformamide, in which the drug was found to be freely soluble. The data are summarized in Table III.

It has been noted that formation of a  $\beta$ -cyclodextrin inclusion complex only slightly increased the equilibrium solubility of chlorothiazide from 0.40 g/L to 0.44 g/L [19]. This observation is consistent with the existence of weak inclusion complexes, characterized by a stability constant of only  $14 \text{ M}^{-1}$ . However, the intrinsic dissolution rate was increased by a factor of 7 with the incorporation of  $\beta$ -cyclodextrin in the solid matrix, which was attributed to a drug carrier effect.

### 3.12 Partition Coefficients

The octanol/water partition coefficient of chlorothiazide has been found to be 0.537 [20], indicating that the drug exhibits little lipophilic character in its neutral state. This value has been found to correlate with the isocratic reversed-phase liquid chromatographic capacity factor of chlorothiazide [20]. The LC parameters have also been found to be more useful in the estimation of drug solubility than were the octanol/water partition coefficients [21].

### 3.13 Ultraviolet Spectrum

UV spectra of chlorothiazide obtained in acidic and basic solution are shown in Figure 4, where it may be noted that the ionized form absorbs at longer wavelengths than does the free acid. Using standard solutions ( $10 \mu\text{g/mL}$ ), the following absorptivity data were obtained [22]:

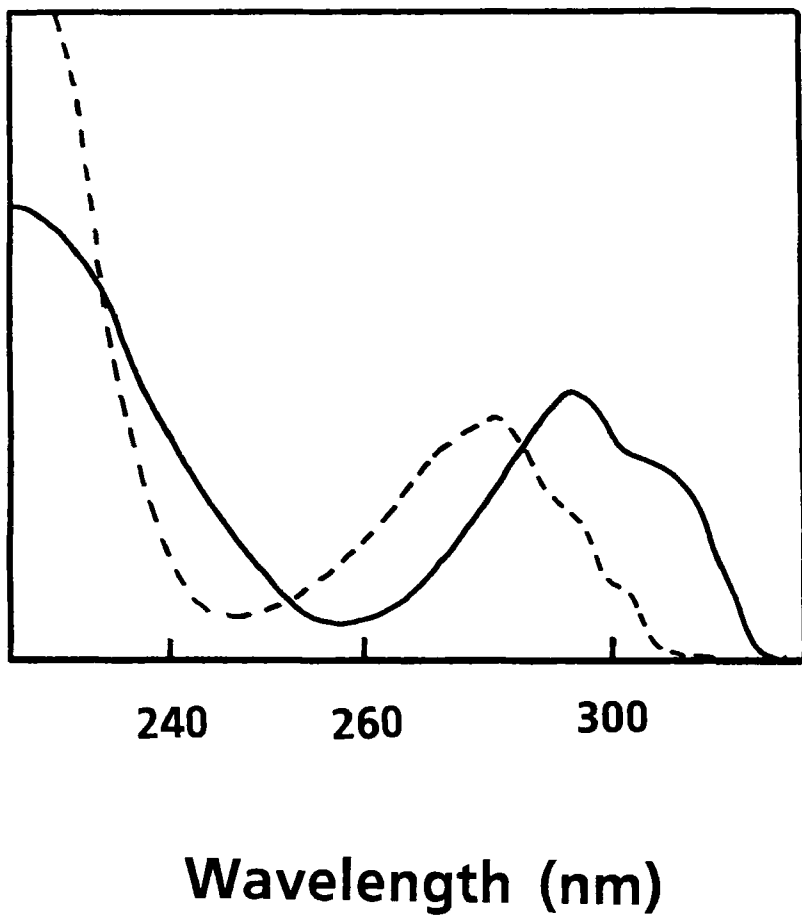
	<u>acidic solution</u>	<u>basic solution</u>
wavelength		
maximum (nm)	280	294
E (1%, 1cm)	580	630

**Table III****Summary of Reported Solubility Data for  
Chlorothiazide**

<u>Solvent</u>	<u>Solubility</u>	<u>Reference</u>
water (pH=4)	0	16
water (pH=7)	0.65 g/L	16
ethanol	1.54 g/L	17
acetone	10 g/L	17
diethyl ether	insoluble	17
chloroform	insoluble	17
benzene	insoluble	17
methanol	slightly soluble	18
pyridine	slightly soluble	18
dimethyl sulfoxide	freely soluble	18
dimethyl formamide	freely soluble	18



Figure 4. Ultraviolet absorption spectrum of chlorothiazide, obtained in acidic (solid trace) and basic (dashed trace) solutions.



### 3.14 Fluorescence Spectrum

Chlorothiazide has been found to be non-fluorescent in acidic solution, but moderately fluorescent in basic solution. The fluorescence excitation maximum was observed at 295 nm, while the fluorescence maximum was found to be 375 nm.

## 4. **Methods of Analysis**

### 4.1 Elemental Analysis

Calculated for  $C_7H_8ClN_3O_4S_2$

	<u>%</u>
C	28.43%
H	2.05%
Cl	11.99%
N	14.21%
O	21.64%
S	21.69%

Found for USP lot  
(after drying)

	<u>%</u>
C	28.38%
H	2.25%
Cl	12.12%
N	14.12%
S	21.57%

### 4.2 Spectrophotometric

The molar absorptivity of chlorothiazide is sufficient so as to permit its direct determination by UV spectroscopy [22,23]. The drug may simply be extracted from crushed tablets using either water or alcoholic solvents, and quantitated through its absorbance [22].

A specific procedure for the determination of benzothiadiazines in dosage forms has been detailed, in which the compounds are derivitized with ethyl acetoacetate [24]. The procedure has been found to be sensitive, quantitative, and specific for this class of compounds. Tablets are pulverized, the chlorothiazide extracted with

dilute NaOH solution, and the extracts acidified with HCl. After dilution to an appropriate level, the diazo derivative of chlorothiazide is generated using nitrite, and then coupled to ethyl acetoacetate. The product thus generated is intensely colored (absorption maximum at 425 nm, and characterized by a molar absorptivity of 34,600), and stable for more than 24 hours. The working analytical range for this method was found to be 2-15  $\mu\text{g/mL}$  for chlorothiazide.

The solution phase fluorescence of chlorothiazide is not sufficiently strong so as to be analytically useful, but the room temperature phosphorescence has been shown to be so [25]. When dissolved in either 1M NaOH or 1M NaOH + 1M NaI and allowed to dry on filter paper, chlorothiazide exhibited room temperature phosphorescence peaking at 440 nm. Detection limits were appropriate for assay of solid dosage forms.

#### 4.3 Chromatographic

##### 4.3.1 Thin-Layer

The determination of chlorothiazide by thin-layer chromatography has been extensively investigated, with this method being primarily used for identity purposes. A large number of solvent systems have been studied for analytical utility, and a selection of these (together with the reported  $R_f$  values) is shown in Table IV. Stohs and Scratchley have also considered various spray reagents for chlorothiazide, and have reported 9 different reagents for which good analytical response was obtained [29].

##### 4.3.2 High Performance Liquid

Liquid chromatography has been the analytical method of choice for the determination of chlorothiazide in body fluids. In one procedure, urine is treated with  $\text{NaBH}_4$  (to convert chlorothiazide to hydrochlorothiazide), acidified with pH 5  $\text{NaH}_2\text{PO}_4$ , and extracted with ethyl

**Table IV**

**Thin-Layer Chromatographic Systems and  
Characteristic  $R_f$  Values Obtained using Silica Gel  
G as the Adsorbant**

<u>Solvent System</u>	<u><math>R_f</math></u>	<u>Reference</u>
80:20 ethyl acetate, benzene	0.17	26
80:20 2-propanol, 12N $\text{NH}_4\text{OH}$	0.70	27
80:20 1-propanol, 12N $\text{NH}_4\text{OH}$	0.85	27
80:20 1-butanol, 12N $\text{NH}_4\text{OH}$	0.26	27
80:20 1-pentanol, 12N $\text{NH}_4\text{OH}$	0.64	27
80:20 chloroform, methanol	0.42	27
90:10 chloroform, methanol	0.10	28
80:20 chloroform, acetone	0.03	28
pure ethyl acetate	0.21	28
pure acetone	0.66	28
50:50 methyl ethyl ketone, n-hexane	0.16	29
70:30 methyl ethyl ketone, n-hexane	0.39	29
60:40 methyl ethyl ketone, n-hexane	0.36	29
50:50 chloroform, acetone	0.31	29

acetate. An absolute recovery of 55% was reported using this procedure. After further washing with pH 8  $\text{NaH}_2\text{PO}_4$ , the ethyl acetate is dried, and re-dissolved in mobile phase (either 8% or 35% acetonitrile in water). Separation and quantitation was obtained on a C18 column [30].

A more direct HPLC method has been proposed, in which hydrochlorothiazide is added to urine samples as an internal standard [31]. The solution is buffered to pH 5.0, and the active compounds extracted with ethyl acetate. The organic layer is taken to dryness, dissolved in methanol, and injected onto the HPLC system. Plasma samples are extracted using toluene, and treated in a similar fashion. Separation was effected on a C8 column, using either aqueous methanol or acetonitrile as the mobile phase.

A variety of HPLC methods have been proposed for the determination of chlorothiazide in body fluids [32-35]. These all use various combinations of extraction procedures and chromatographic conditions to effect isolation and separation.

#### 4.4 Electrochemical

Chlorothiazide has been found to be electrochemically active, with its reduction being observable in two waves [36]. In borate buffer (pH 8.1), the  $E_{1/2}$  values were found to be -1.65 and -1.88 volts. In N,N-dimethylformamide solution, the  $E_{1/2}$  values were determined to be -1.12 and -1.45 volts. The electrochemistry of chlorothiazide was used to develop a method for its determination in solid dosage forms, since most tablet constituents did not appreciably affect the reduction potentials [37].

In a detailed study, the use of conventional DC polarography was contrasted with differential pulse polarography (DPP) for quantitation of chlorothiazide in solid dosage forms [38]. The DPP method was found to yield superior analytical results, and applicable even in instances where more than one electroactive substance was present.

## 5. Stability

### 5.1 Solid Stability

Chlorothiazide is stable in the solid state, undoubtedly due to its anhydrous nature. Long term storage in airtight containers has been recommended, however. The sodium salt is reported to be equally stable as the free acid in the solid state.

### 5.2 Solution Stability

Chlorothiazide (free acid) was found to be stable in an aqueous suspension at pH 6.5 and less. The compound is not stable in alkaline solutions, and undergoes hydrolytic decomposition upon standing.

### 5.3 Incompatibilities

Chlorothiazide forms a precipitate upon mixing with aqueous solutions of hydrallazine and reserpine, but not with protoveratrine A and B, pentolinium tartrate, cryptenamine acetate, alkavervir, and mecamlamine hydrochloride [39]. Chlorothiazide also forms a precipitate upon mixing with aqueous chlorpromazine hydrochloride, promazine hydrochloride, or promethazine hydrochloride [40].

### 5.4 Stability in Biological Fluids

Chlorothiazide does not appear to exhibit instability in biological fluids, and no special precautions are usually taken to preserve either urine, blood, or plasma samples.

## 6. Drug Metabolism, Pharmacokinetics

Chlorothiazide is used clinically for the treatment of hypertension, congestive heart failure, and edematous conditions. It enhances

the renal excretion of sodium and an accompanying volume of water, resulting in diuresis. As with other thiazides, chlorothiazide is excreted unchanged in urine, and no metabolism products have been reported [3]. The drug also exerts a mild antihypertensive effect in hypertensive animals and patients, but does not appear to lower the blood pressure of normal subjects [5].

When used as an antihypertensive, chlorothiazide is administered orally in 250 mg doses 3 times daily. The diuretic activity requires higher doses, with the dose being 500 mg twice daily. When orally administered, chlorothiazide produces a response in about 2 hours, with the diuresis being maintained for 6 to 12 hours. Tolerance does not develop, and therapeutic efficacy can be maintained over long periods. However, continuous therapy is not desirable, and maintenance doses are adjusted to keep the patient free from oedema.

Problems with the pharmacology of chlorothiazide ultimately led to its replacement by other diuretic agents, primarily its closely related analogue hydrochlorothiazide. The adsorption of orally administered chlorothiazide has been shown by urinary excretion data to be incomplete and variable [41]. Over 90% of intravenous chlorothiazide is recovered unchanged in urine, while the recovery from orally administered doses is less than 25%. The available data suggests the lack of dose proportional adsorption, as well as drug elimination [42].

## 7. Acknowledgement

Special thanks are due to Dr. G. Brewer, for his assistance during the initial stages of the literature searching.

## 8. References

1. K.H. Beyer, *Perspect. Biol. Med.*, 20, 410 (1977).
2. K.H. Beyer, *Br. J. Clin. Pharmac.*, 13, 15 (1982).
3. L.S. Goodman and A. Gilman, The Pharmacological Basis of Therapeutics, Macmillan Pub. Co., New York, 1975, pp. 825-830.
4. R.F. Pitts, "Sulfonamyl Diuretics", chapter 17 in The Physiological Basis of Diuretic Therapy, Charles Thomas, Pub., Springfield, Illinois, 1959.
5. J.G. Topliss, "Diuretics", chapter 38 in Medicinal Chemistry, 3<sup>rd</sup> edition, A. Burger, ed., Wiley-Interscience, New York, 1970.
6. F.C. Novello and J.M. Sprague, *J. Am. Chem. Soc.*, 79, 2028 (1957).
7. F.C. Novello, S.C. Bell, E.L.A. Abrams, C. Ziegler, and J.M. Sprague, *J. Org. Chem.*, 25, 965, 970 (1960).
8. P. Jakobsen and S. Treppendahl, *Tetrahedron*, 35, 2151 (1979).
9. A.F. Casy, *J. Pharm. Biomed. Anal.*, 5, 247 (1987).
10. I. Sunshine, in Handbook of Mass Spectra of Drugs, CRC Press, Boca Raton, Florida, 1981.
11. W.H. De Camp, *J. Assoc. Off. Anal. Chem.*, 67, 927 (1984).
12. C.W. Whitehead, J.J. Traverso, H.R. Sullivan, and D.E. Morrison, *J. Org. Chem.*, 26, 2809 (1961).
13. A. Essig, *Am. J. Physiol.*, 201, 303 (1961).



14. W.F. Charnicki, F.A. Bacher, S.A. Freeman, and D.H. DeCesare, J. Am. Pharm. Assoc., Sci. Ed., 48, 656 (1959).
15. U.G.G. Hennig, L.G. Chatten, R.E. Moskalyk, and C. Ediss, Analyst, 106, 557 (1981).
16. "The Merck Index", 10<sup>th</sup> edition, M. Windholz and S. Budavari, eds., Merck & Co., Rahway, NY 1983.
17. "Martindale, The Extra Pharmacopoeia", J.E.F. Reynolds, ed., 28<sup>th</sup> edition, The Pharmaceutical Press, London, 1977.
18. "Remington's Pharmaceutical Sciences", A.R. Gennaro, ed., 17<sup>th</sup> edition, Mack Publishing Co., Easton, PA, 1985.
19. O.I. Corrigan and C.T. Stanley, J. Pharm. Pharmacol., 34, 621 (1982).
20. T.L. Hafkenschied and E. Tomlinson, Int. J. Pharm., 16, 225 (1983).
21. T.L. Hafkenschied and E. Tomlinson, Int. J. Pharm., 17, 1 (1983).
22. V.B. Pilsbury and J.V. Jackson, J. Pharm. Pharmacol., 18, 713 (1966).
23. D.K. Argawal and A.V. Deshpande, Pharmazie, 37, 150 (1982).
24. F. Belal, M. Rizk, F. Ibrahiem, and M.S. El-Din, Talanta, 33, 170 (1986).
25. E.L.Y. Bower and J.D. Winefordner, Anal. Chim. Acta, 101, 319 (1978).
26. R. Adam and C.L. Lapiere, J. Pharm. Belg., 19, 82 (1964).
27. P.J. Smith and T.S. Hermann, Anal. Biochem., 22, 134 (1968).
28. P. Owen, A. Pendlebury, and A.C. Moffat, J. Chrom., 161, 195 (1978).

29. S.J. Stohs and G.A. Scratchley, J. Chrom., 114, 329 (1975).
30. P.A. Tisdall, T.P. Moyer, and J.P. Anhalt, Clin. Chem., 26, 702 (1980).
31. R.H. Barbhuiya, T.A. Philips, and P.G. Welling, J. Pharm. Sci., 70, 291 (1981).
32. V.P. Shah, J. Lee, and V.K. Prasad, Anal. Lett., 15, 529 (1982).
33. G.A. Hessey, M.L. Costanzer, and W.F. Bayne, J. Chrom., 380, 450 (1986).
34. R.O. Fullinfaw, R.W. Bury, and R.F.W. Moulds, J. Chrom., 415, 347 (1987).
35. R.M. Smith, G.A. Murilla, T.G. Hurdley, R. Gill, and A.C. Moffat, J. Chrom., 384, 259 (1987).
36. A.I. Cohen, B.T. Keeler, N.H. Coy, and H.L. Yale, Anal. Chem., 34, 216 (1962).
37. E. Kkolos and J. Walker, Anal. Chim. Acta, 80, 17 (1975).
38. C. van Kerchove, R. Bontemps, and A. Schoenmakers, J. Pharm. Pharmacol., 34, 420 (1982).
39. W.F. Charnicki, J. Am. Pharm. Assoc., Sci. Ed., 48, 656 (1959).
40. B.B. Riley, J. Hosp. Pharm., 28, 228 (1970).
41. M.C. Meyer, "Final Progress Report - Chlorothiazide Bioavailability Study", Food and Drug Administration, Contract 223-74-3097 (1976).
42. V.P. Shah, J. Lee, J.P. Hunt, V.K. Prasad, B.E. Cabana, and T. Foster, Curr. Therap. Res., 29, 823 (1981).

# CLIOQUINOL

Gandharva Padmanabhan, Ingrid Becue, James B. Smith

Drug Development Department  
Pharmaceuticals Division  
CIBA-GEIGY Corporation  
Suffern, NY 10901

## 1. Description

- 1.1 Name, Formula, Molecular Weight
- 1.2 Appearance

## 2. Physical and Chemical Properties

- 2.1 Infrared Absorption Spectrum
- 2.2 Nuclear Magnetic Resonance Spectrum
- 2.3 Ultraviolet Absorption Spectrum
- 2.4 Mass Spectrum
- 2.5 Optical Rotation
- 2.6 Melting Range
- 2.7 Differential Scanning Calorimetry
- 2.8 Thermogravimetric Analysis
- 2.9 Solubility
- 2.10 X-Ray Diffraction
- 2.11 Polymorphism
- 2.12 Partition Coefficient
- 2.13 Dissociation Constant
- 2.14 Complexation

## 3. Synthesis

## 4. Stability - Degradation

- 4.1 Solution
- 4.2 Solid State

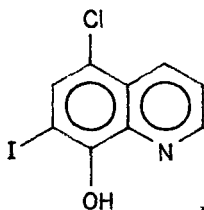
5. Drug Metabolism and Pharmacokinetics
6. Toxicity
7. Methods of Analysis
  - 7.1 Identification
  - 7.2 Elemental Analysis
  - 7.3 Titrations
  - 7.4 Phase-Solubility Analysis
  - 7.5 Thin-Layer Chromatography
  - 7.6 Gas Chromatography
  - 7.7 High Pressure Liquid Chromatography
  - 7.8 Infrared Absorption Spectrophotometry
  - 7.9 Ultraviolet Absorption Spectrophotometry
  - 7.10 Colorimetric Analysis
  - 7.11 Polarography
  - 7.12 X-Ray Fluorescence Analysis
  - 7.13 Gravimetric Methods

## References

### 1. DESCRIPTION

#### 1.1 Name, Formula, Molecular Weight

Clioquinol, also known as iodochlorhydroxyquin and iodochloroxyquin, is 5-chloro-7-iodo-8-quinolinol or 5-chloro-7-iodo-8-hydroxyquinoline.



$C_9H_5ClINO$

Molecular Weight: 305.5

#### 1.2 Appearance

Clioquinol occurs as a white to yellowish white, light, voluminous, spongy powder with a very faint characteristic odor.

## 2. PHYSICAL AND CHEMICAL PROPERTIES

### 2.1 Infrared Absorption Spectrum

The infrared spectra obtained with a mineral oil suspension and a potassium bromide pellet are shown in Figures 1 and 2. The spectral assignments for the nujol mull spectrum are listed in Table I.

Table I

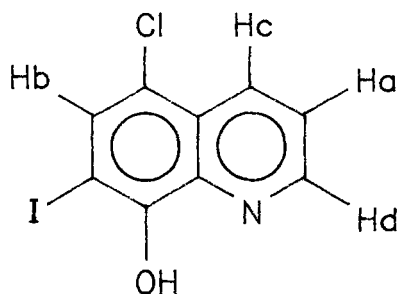
Wavenumber, $\text{cm}^{-1}$	Assignment
785	Three adjacent H's in aromatic
1580, 1610	Aromatic, C=C, C=N
1205, 3140	Phenolic OH
960	Aromatic chloro

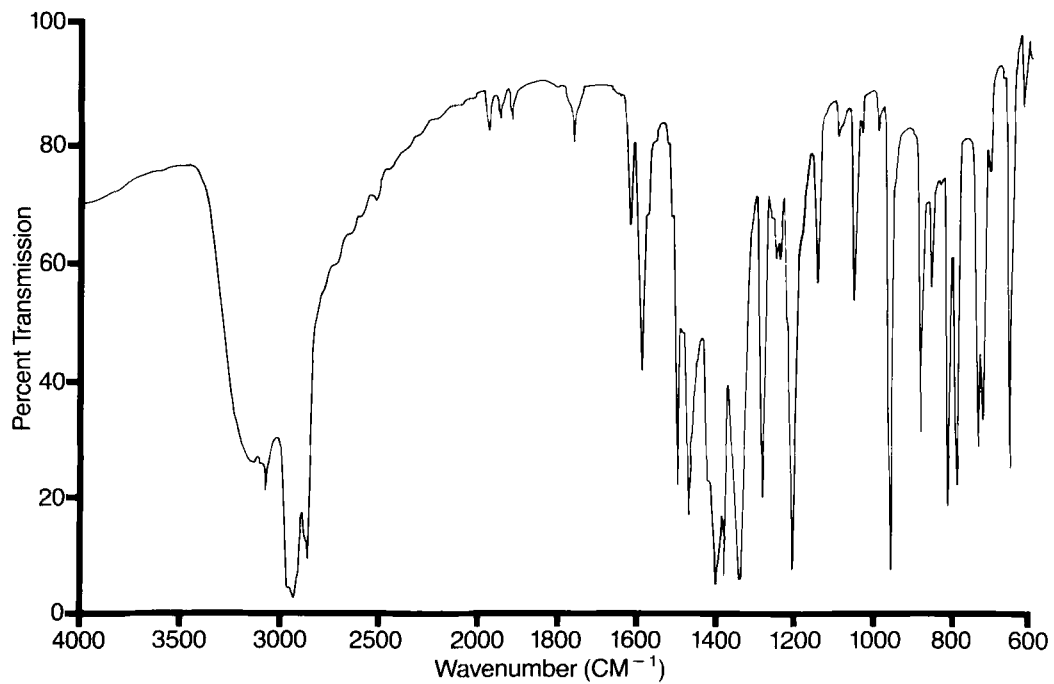
### 2.2 Nuclear Magnetic Resonance Spectra (NMR)

#### 2.2.1 Proton Spectrum

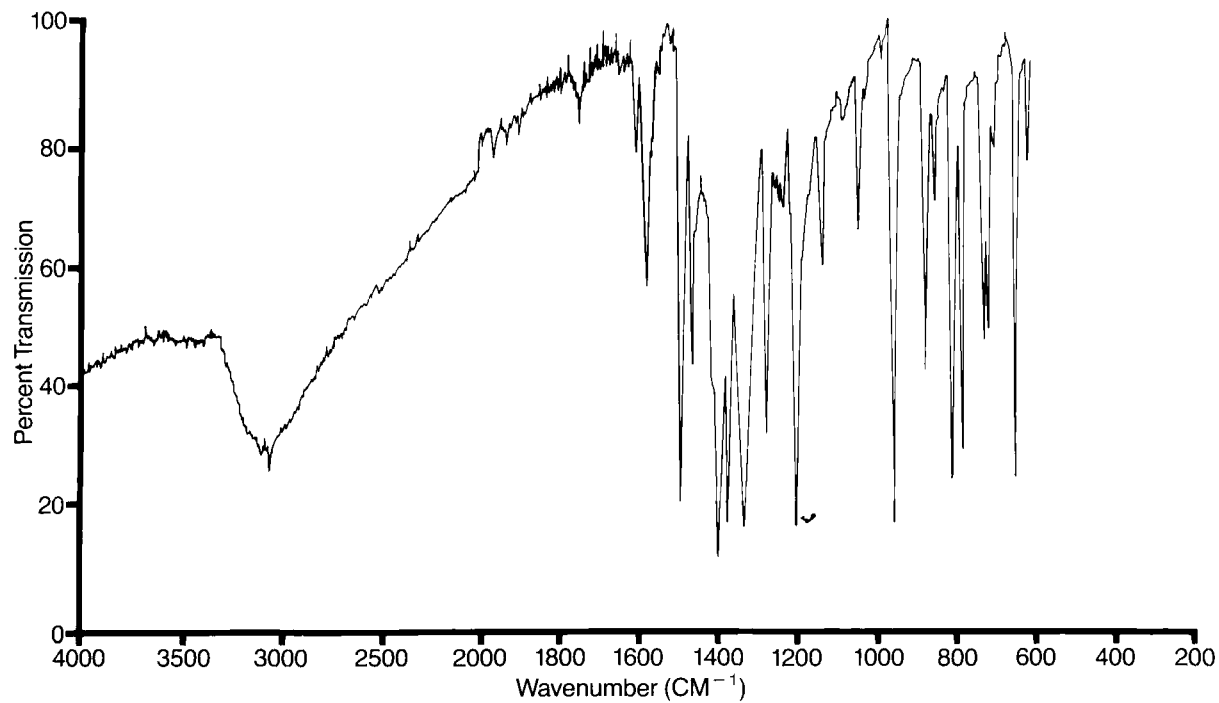
The proton NMR spectrum of clioquinol is shown in Figure 3. The spectrum was obtained with a Perkin-Elmer R-24B 60 MHz spectrometer at ambient temperature. The sample was dissolved in deuterated dimethyl sulfoxide containing tetramethylsilane as an internal standard. The spectral assignments are shown in Table II.

Table II

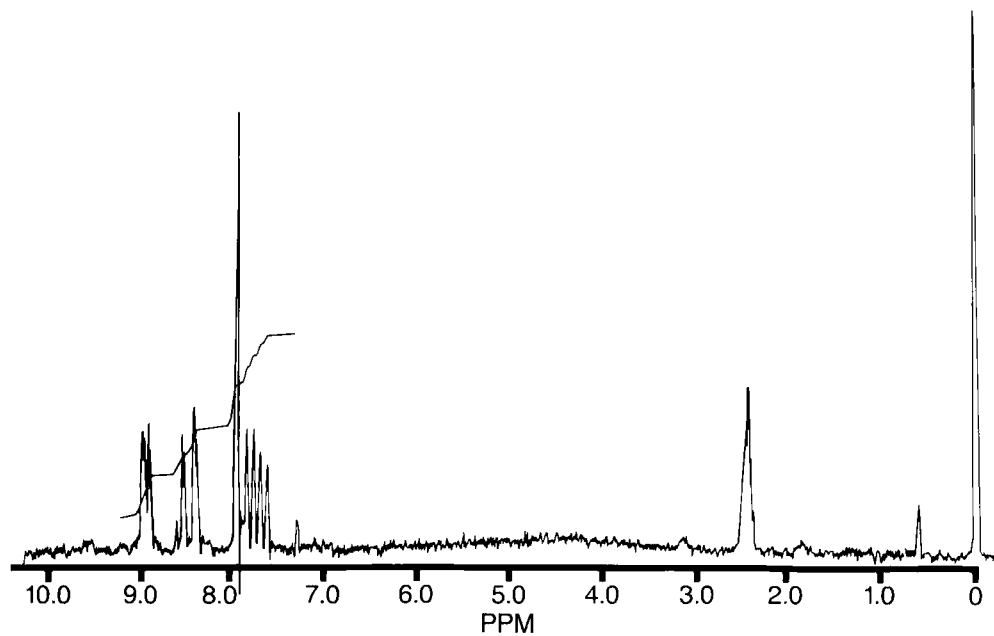




**Figure 1:**  
**Infrared Absorption Spectrum of Clioquinol in Mineral Oil**



**Figure 2:**  
**Infrared Absorption Spectrum of Clloquinol in KBr**



**Figure 3:**  
**Proton NMR Spectrum of Clioquinol in Dimethyl Sulfoxide**



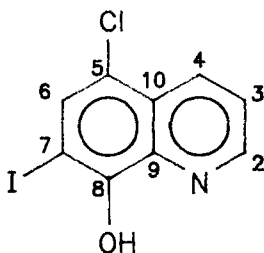
Table II (continued)

Chemical Shift (ppm)	Multiplicity	Number of Protons	Assignment
7.6-8.0	Multiplet	1	a
8.0	Singlet	1	b
8.4-8.7	Multiplet	1	c
8.9-9.1	Multiplet	1	d

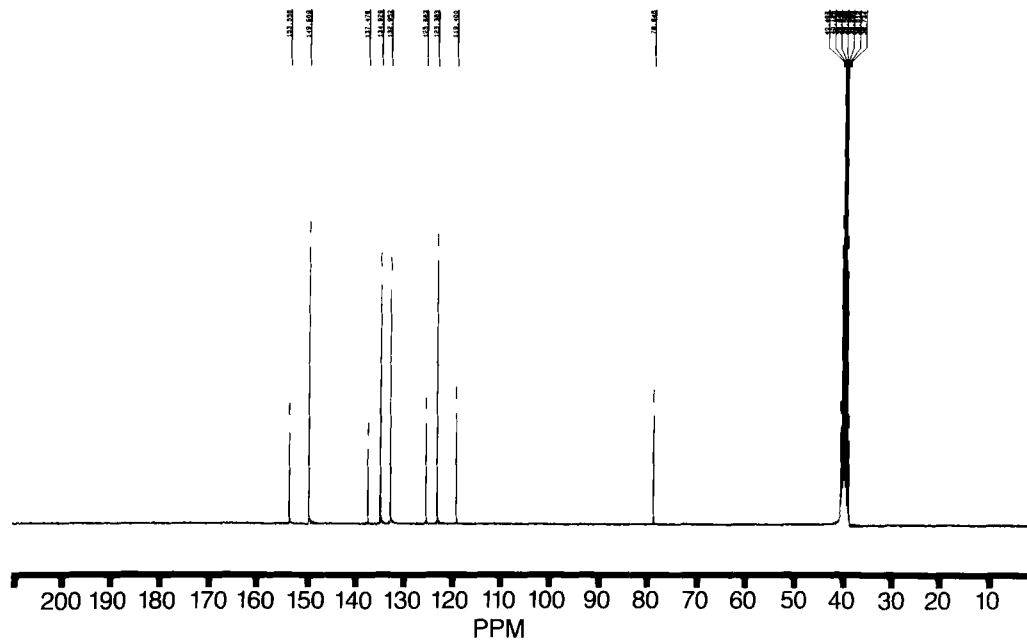
## 2.2.2 Carbon-13 Spectrum

The carbon-13 NMR spectrum of clioquinol is shown in Figure 4. The spectrum was obtained on a Bruker AM-300, 300 MHz spectrometer with sample dissolved in deuterated dimethyl sulfoxide. The spectral assignments are shown in Table III.

Table III



Chemical Shift, ppm	Assignment
78.845	7
119.400	5
123.383	3
125.663	10
132.959	4
134.929	6



**Figure 4:**  
**Carbon-13 NMR Spectrum of Clioquinol in Deuterated Dimethyl Sulfoxide**

Table III (continued)

Chemical Shift, ppm	Assignment
137.476	9
149.603	2
153.558	8

### 2.3 Ultraviolet Absorption Spectrum

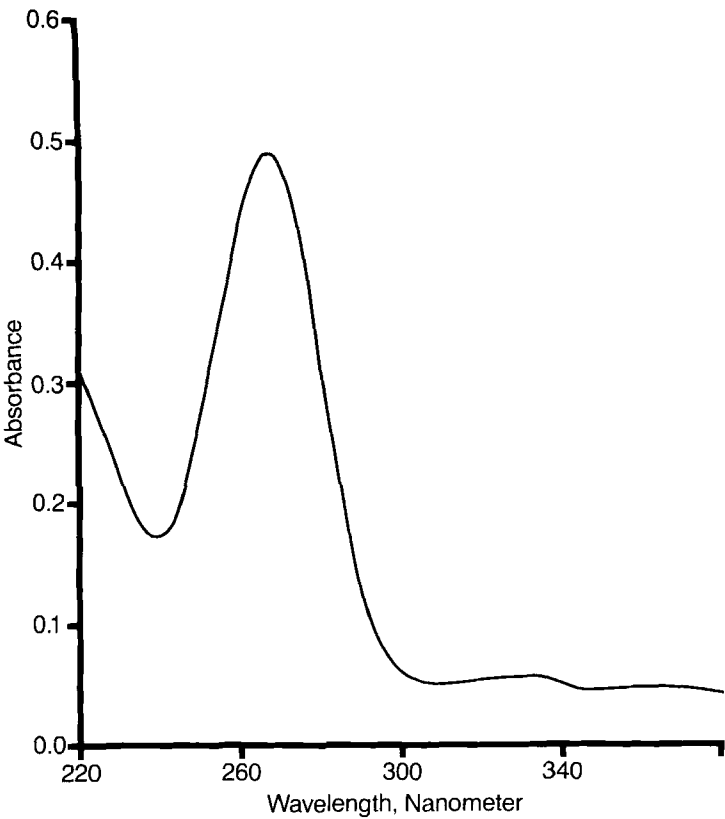
The ultraviolet absorption spectra of clioquinol solutions are shown in Figures 5, 6 and 7. A 5  $\mu\text{g/mL}$  solution in 3:1 water/concentrated hydrochloric acid (Figure 5) exhibits a maximum at 266 nm with an  $A(1\%,1\text{cm})$  value of 990. A 5  $\mu\text{g/mL}$  solution in 0.1N methanolic sodium hydroxide exhibits (Figure 6) a maximum at 269 nm with an  $A(1\%,1\text{cm})$  value of 1120. Ethanolic solution of the drug containing 6  $\mu\text{g/mL}$  exhibited maximum at 255 nm (Figure 7) with an  $A(1\%,1\text{cm})$  value of 1570.

### 2.4 Mass Spectrum

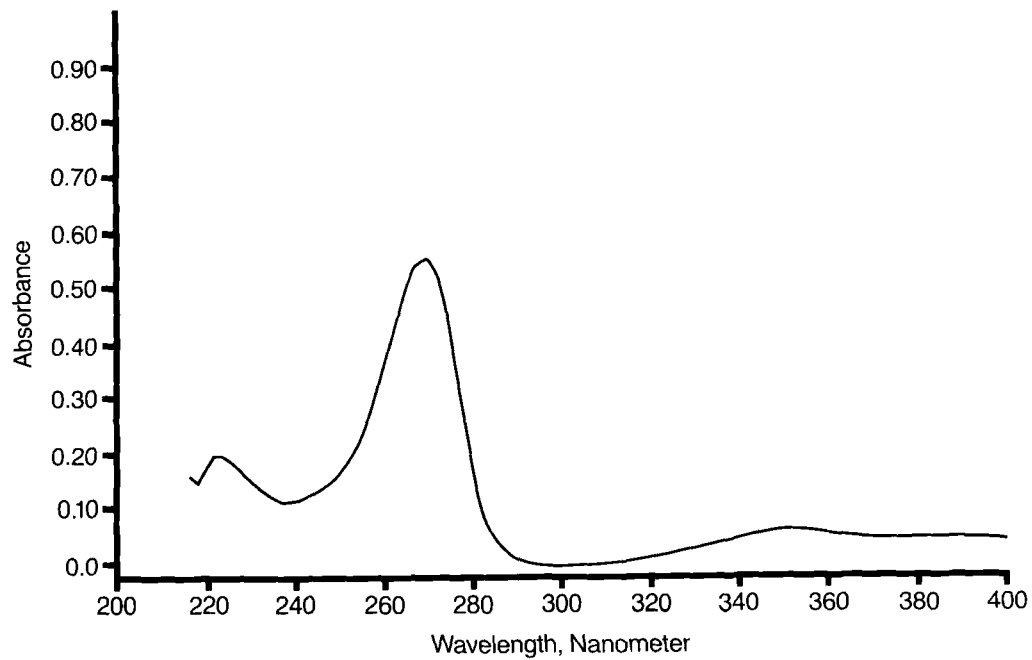
The low resolution electron impact mass spectrum of clioquinol obtained at 70 ev using a solid probe insertion is shown in Figure 8. The spectrum was run on a Kratos MS 25 spectrometer interfaced with a data handling system. The prominent fragments and their mass/charge ratios are illustrated in Table IV.

Table IV

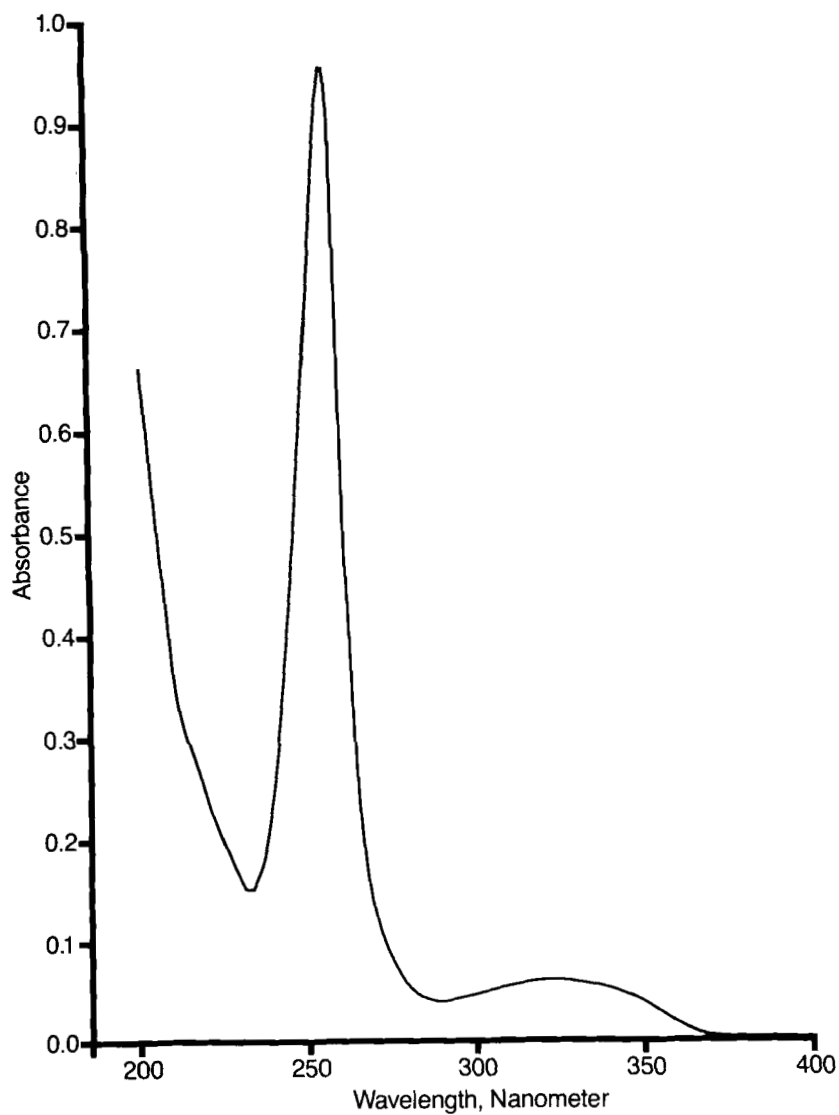
m/e	Fragment
305, 307	$M^+$
277, 279	$[M-CO]^+$
178, 180	$[M-I]^+$
150, 152	$[M-CO-I]^+$



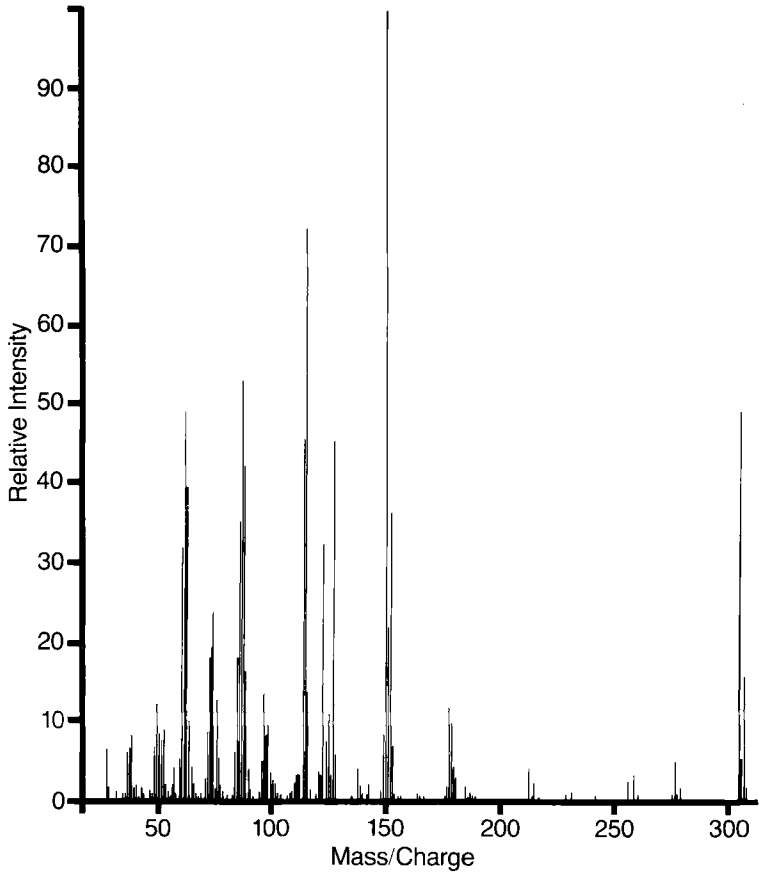
**Figure 5:**  
**Ultraviolet Absorption Spectrum of Clioquinol in 1:4 HCl**



**Figure 6:**  
**Ultraviolet Absorption Spectrum of Clioquinol in 0.1N Methanolic Sodium Hydroxide**



**Figure 7:**  
**Ultraviolet Absorption Spectrum of Clioquinol in Ethanol**



**Figure 8:**  
**Low Resolution Electron Impact Mass Spectrum of Clioquinol**

Table IV (continued)

m/e	Fragment
123, 125	[M-CO-I-HCN] <sup>+</sup>
115	[M-CO-I-Cl] <sup>+</sup>

## 2.5 Optical Rotation

The molecule of clioquinol has no chiral center and hence the drug does not exhibit any optical activity.

## 2.6 Melting Range

Clioquinol melts between 177°C and 179°C with decomposition when tested according to the USP XXI, Class Ia procedure. A value of 182°C has been reported for pure clioquinol (1).

## 2.7 Differential Scanning Calorimetry (DSC)

The DSC thermogram of clioquinol shows a single melt endotherm between 182°C and 190°C. The peak temperature for the melting with decomposition occurs at approximately 185°C. The thermogram, shown in Figure 9, was obtained with a Dupont Model 900 instrument at a scan rate of 10°C/minute.

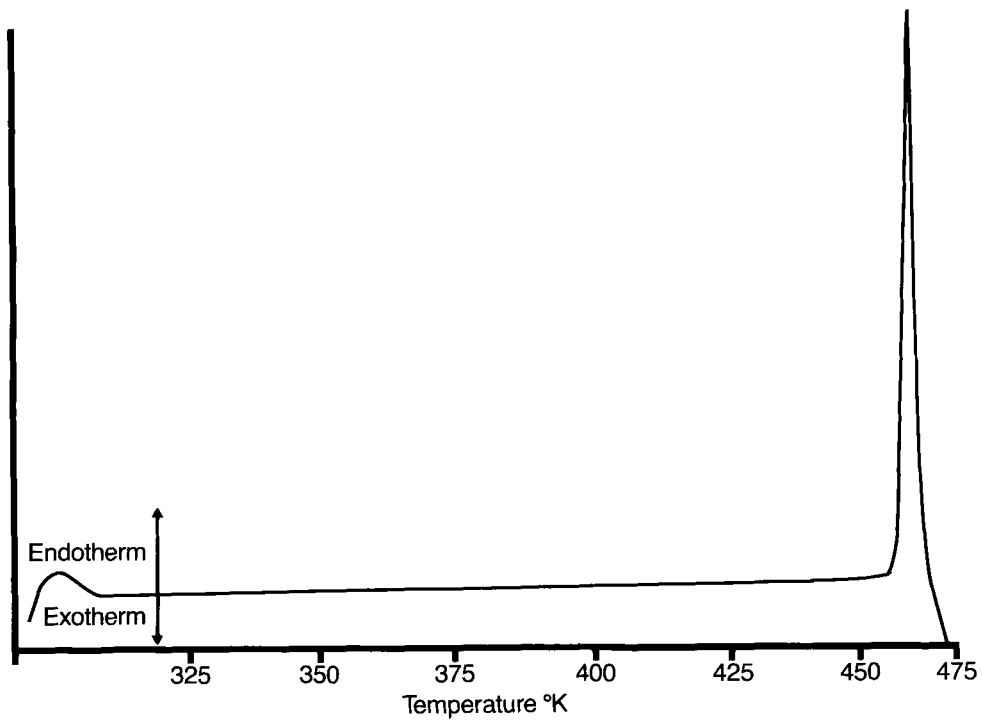
## 2.8 Thermogravimetric Analysis (TA)

The TA of clioquinol exhibited a weight loss of 0.07% between room temperature and 120°C. A rapid rate of weight loss was observed above 120°C.

## 2.9 Solubility

The solubilities of clioquinol in different solvents were determined after equilibrating 0.5 g of the sample in 25 mL of solvent at the temperature indicated in Table V.





**Figure 9:**  
**DSC Scan of Clioquinol**

Table V

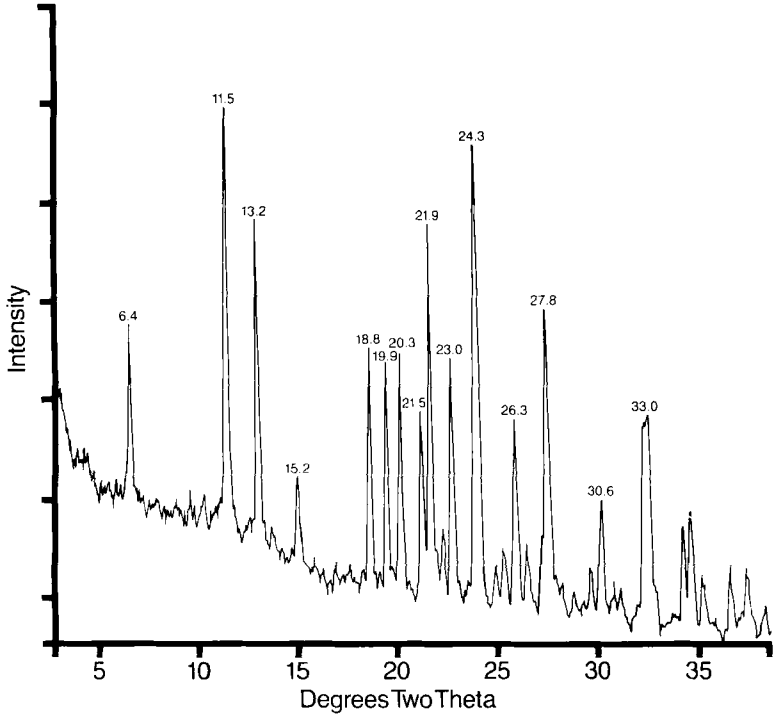
Solvent	Temperature	Solubility, mg/mL
Water	Room Temperature	<0.01
Methanol	Room Temperature	1.9
Ethanol	Room Temperature	1.3
Ether	Room Temperature	5.4
Chloroform	Room Temperature	14.9
0.1N NaOH	Room Temperature	17.3
0.1N HCl	Room Temperature	0.02
Intestinal Fluid	37°C	<0.01
Gastric Fluid	37°C	0.02
Acetonitrile	Room Temperature	>1.7 <2.5
Tetrahydrofuran	Room Temperature	>20 <100
Ethyl Acetate	Room Temperature	>6.7 <10
Carbon Disulfide	Room Temperature	>10 <20
Dimethyl Sulfoxide	Room Temperature	>20 <100
Dimethyl Acetamide	Room Temperature	>20 <100

### 2.10 X-Ray Diffraction

The x-ray powder diffraction pattern obtained for clioquinol is shown in Figure 10. The data were collected on a GE Model XRD-spectrogoniometer using a  $\text{CuK}_\alpha$  (1.542Å) with a Ni filter as a radiation source.

### 2.11 Polymorphism

No polymorphism has been reported for clioquinol.



**Figure 10:**  
**X-ray Powder Diffraction Pattern of Clioquinol**

### 2.12 Partition Coefficient

The following partition coefficient data for clioquinol were obtained when 25 mL of 0.1 mg/mL and 1 mg/mL solutions in the appropriate organic solvents were partitioned individually with 25 mL of the indicated aqueous solutions at room temperature.

Table VI

Aqueous Phase	Organic Phase	Partition Coefficient*
0.1N HCl	Chloroform	$\rightarrow \infty$
pH 7 Buffer	Chloroform	$\rightarrow \infty$
0.1N NaOH	Chloroform	0.36
0.1N HCl	Ether	$\rightarrow \infty$
pH 7 Buffer	Ether	$\rightarrow \infty$
0.1N NaOH	Ether	$\rightarrow 0$
pH 7 Phosphate Saline Buffer	n-Decane	1750(2)

\*Concentration in organic phase/concentration in aqueous phase

### 2.13 Dissociation Constant

Clioquinol can ionize in solutions both as an acid and as a base. The dissociation constants have been reported in the literature (2) based on the partition of clioquinol (or tritiated clioquinol) between n-decane and buffers of appropriate pH values and determination of clioquinol by spectrophotometry (or scintillation counting). The buffers employed were generally 0.02 M in phosphate and they also contained 0.13 M sodium chloride and 0.5 mM EDTA. The values reported for pK<sub>a</sub> were 3.17 for the deprotonation of the protonated nitrogen and 8.07 for the deprotonation of the phenolic group. The pK<sub>a</sub> value for the phenolic group has also been reported to be 8.12 based on the potentiometric (3) titration at 35°C with 50:50 (v/v) ethanol/water as solvent.

## 2.14 Complexation

8-Hydroxyquinoline is a well known complexing agent (4, 5, 6) and hence clioquinol can also be expected to form complexes with metal ions. The following formation constant values for clioquinol have been reported in the literature (3).

Table VII

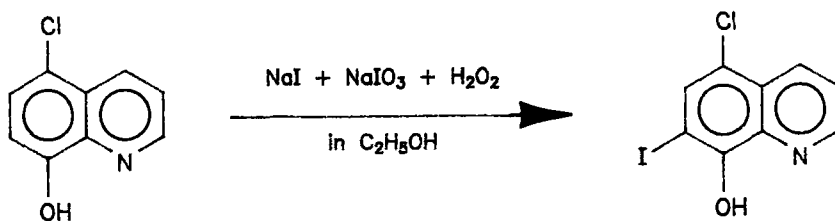
Metals Ions	$\log K_1$	$\log K_2$	$\log K$
$\text{Cu}^{2+}$	8.85	6.95	15.80
$\text{Zn}^{2+}$	6.99	5.48	12.47
$\text{Mn}^{2+}$	5.48	4.00	9.48
$\text{Mg}^{2+}$	4.95	3.60	8.55
$\text{Ca}^{2+}$	4.85	3.28	8.13

$$K_1 = [\text{MLi}]/[\text{M}][\text{Li}]; K_2 = [\text{MLi}_2]/[\text{MLi}][\text{Li}];$$

$$K = K_1 K_2 [\text{MLi}_2]/[\text{M}][\text{Li}]^2$$

## 3. SYNTHESIS

Clioquinol is synthesized by iodination of 5-chloro-8-hydroxyquinoline hydrochloride by the following reaction (6):



The major impurity likely to be present in clioquinol active ingredient is the precursor, 5-chloro-8-hydroxyquinoline. Other potential impurities are 5,7-diiodo-8-hydroxyquinoline, 5,7-dichloro-8-hydroxyquinoline and 7-chloro-8-hydroxyquinoline.

#### 4. STABILITY-DEGRADATION

##### 4.1 Solution

Clioquinol was found to undergo significant degradation in acidic, basic and photolyzed acetonitrile solutions. The acidic (0.05% w/v in 4N HCl) and basic (0.05% w/v in 0.1N NaOH) solutions were analyzed during a 2 day reflux period by a gas-liquid chromatographic (GLC) procedure. For photolysis, 0.05% w/v solution in acetonitrile was exposed to 600 foot-candle light source for 6 days and samples analyzed periodically again by GLC

Time	% Clioquinol Remaining		
	Refluxed 0.1N NaOH	Refluxed 4N HCl	Photolyzed Acetonitrile Solution
0	100	100	100
2.5 Hours	N.D.	76	98
5 Hours	90	N.D.	98
1 Day	54	65	92
2 Days	32	34	80
3 Days	N.D.	N.D.	63
6 Days	N.D.	N.D.	39

N.D. = Not Determined

The major degradation compound under both acidic and basic hydrolysis was identified to be 5-chloro-8-hydroxy-quinoline. No attempt was made to identify the photolytic degradation compound.

##### 4.2 Solid State

Samples of clioquinol were stored under air, nitrogen, oxygen, 35°C/85% relative humidity and 600 foot-candle light source and analyzed by GLC. The samples exposed to air, nitrogen and oxygen atmospheres were maintained at 105°C for one month. For 35°C/85% relative humidity and 600 foot-candle

photolytic conditions, samples were stored in open weighing bottles for one month. Results from GLC analysis indicated no significant degradation of clioquinol in any of the storage conditions.

## 5. DRUG METABOLISM AND PHARMACOKINETICS

When human volunteers were given (7) single oral dose of 250, 750 and 1500 mg of the drug in the powder form, no free clioquinol was detected in urine samples (8, 9). However, evidence for the presence of less than 1% of conjugates of clioquinol was obtained which indicates that clioquinol is absorbed extensively in the GI tract. Calculation of the area under the curve for blood plasma samples, determined by a GLC method, indicates a half-life of 11-14 hours with maximum concentration occurring at 4 hours.

Multiple dose human studies (7), carried out by administering orally 3 x 250 mg daily for 7 days, showed an equilibrium between the amount absorbed and the amount eliminated after the fifth day of administration. Three days after the discontinuation of the drug, the amount of clioquinol was below the limit of detection.

Results from skin absorption studies (10) employing topical application of clioquinol in cream and ointment formulations indicate that the drug is absorbed rapidly from human skin. A mean serum concentration in the range of 0.3-1.3  $\mu\text{g/mL}$  was reached the second day of treatment and this range persisted throughout the topical treatment.

## 6. TOXICITY

An oral  $\text{LD}_{50}$  value of 69 mg/kg in male mice has been reported for clioquinol (6). Other reported  $\text{LD}_{50}$  values are:  $>1.99 \text{ g/m}^3/4\text{H}$  for inhalation for male rats and  $>3.04 \text{ kg/kg}$  for dermal route for rabbits.

## 7. METHODS OF ANALYSIS

### 7.1 Identification

Three identity tests are given in USP XXI for clioquinol based on the following: ultraviolet absorption maximum at 267 nm, a test for liberated iodine and a gas chromatographic retention time.

## 7.2 Elemental Analysis

The following elemental composition was obtained when a sample was analyzed by Perkin Elmer Model 2400 CHN Analyzer for C, H, and N and by Schöniger combustion/silver nitrate titration for Cl and I.

Element	Theory, %	Found, %
Carbon	35.38	35.4
Hydrogen	1.65	1.7
Nitrogen	4.59	4.6
Chlorine	11.60	11.3
Iodine	41.54	43.1

## 7.3 Titrations

### 7.3.1 Non-aqueous Titration

Clioquinol can be titrated in glacial acetic acid with perchloric acid in glacial acetic acid as titrant. The titration can be carried out potentiometrically using a glass calomel electrode containing lithium chloride saturated glacial acetic acid. Clioquinol can also be titrated as an acid in pyridine or dimethylformamide as solvents with methanolic sodium hydroxide as titrant.

### 7.3.2 Silver Nitrate Titration of Liberated Halides

The chloride and iodide ions liberated from clioquinol, after Schöniger combustion and reduction with hydrazine sulfate, can be titrated potentiometrically using a silver electrode and a mercurous sulfate-potassium sulfate reference electrode. Iodine and chlorine are quantitated based respectively on the first and second end-points.

## 7.4 Phase Solubility Analysis

Phase solubility analysis of clioquinol has been carried out using acetone (approximate solubility ~7.5 mg/mL at 25°C) as solvent (6).

## 7.5 Thin-Layer Chromatography

Several thin-layer chromatographic systems have been employed for the identification of clioquinol and the estimation of related compounds in active drug (6).



System I

Adsorbent: Machery Nagel Precoated  
20 x 20 cm Polyamide II UV254 plates,  
0.2 mm thickness

Mobile Phase: Methanol/Acetic Acid (19:1)

Detection System: Longwave UV.

System II

Adsorbent: Silica Gel H (Merck) containing  
citric acid (6)

Mobile Phase: Chloroform

Detection: Sprayed with ethanolic solution of  
4-methylumbelliferone, exposed to  
ammonia vapor and observed under  
visible and long wave UV.

System III

Adsorbent: Polyamide (Woelm) powder with  
calcium sulfate coated on a glass  
plate (11)

Mobile Phase: Methanol

Detection System: 1. UV at 266 nm  
2. Pauly reagent spray

System IV

Adsorbent: Silcia Gel

Mobile Phase: Methanol/methoxyethanol/hydrochloric  
acid (88:10:2). Developed three  
times (12)

Detection Information not available

System V

Adsorbent: Silica Gel 60 HR containing  
fluorescence indicator F254 and  
pH 5.7 phosphate buffer coated on a  
plate to 250  $\mu$  thickness (13)

Mobile Phase: Triethylamine/Dioxane/Methylethyl  
Ketone (80:15:5). Develop three  
times

- Detection:
1. Shortwave UV (254 nm)
  2. Extraction of silica with acidified methanol and quantitation by spectrophotometry at 269 nm

## 7.6 Gas Chromatography

### System I

The following system has been employed for the analysis of the drug in the active ingredient formulation (14):

Column: 3% OV-17 on Gas Chrom Q  
(80-100 mesh) 6 feet x 2 mm glass column.

Temperature: Injector at 170°C. Detector at 250°C. Column at 160°C

Carrier: Helium, 30 mL/minute

Detector: Flame Ionization

Internal Standard: Pyrene

Sample

Derivatization: Derivatized with N,O-bis(trimethylsilyl)acetamide (BSA) in 4:1 pyridine/n-hexane. Instead of the OV-17 column, 3% OV-101 on Gas Chrom Q has also been employed to analyze only the active drug employing the conditions described above.

### System II

The following systems have been employed for the determination of the drug substance in feed mixes (15).

Column: 5% JXR, on Gas Chrom Q (100-120 mesh) 5 feet x 4 mm i.d. glass column.

Temperature: Injector at 200°C. Detector information not available. Column at 180°C

Carrier: Nitrogen, 80 mL/minute

Detector: Flame Ionization; H<sub>2</sub> - 80 mL/minute, Air - 600 mL/minute

Internal Standard: Eicosane

Sample: Derivatized with BSA at room temperature in dichloromethane and again in carbon disulfide

### System III

Column: 16% OV-17 on Gas Chrom Z (100-120 mesh) or 10% OV-17 on Gas Chrom Q (100-120 mesh) on 5 feet x  $\frac{1}{4}$  inch i.d. glass column (16)

Temperature: Injector at 210-220°C. Detector information not available. Column at 200°C

Carrier: Nitrogen, 60 mL/minute

Detector: Flame Ionization

Internal Standard: Eicosane

Sample: Derivatized with BSA in dichloromethane/dimethoxypropane mixture as solvent at room temperature and then the solvent is evaporated.

### System IV

The following all glass system has been employed for the analysis of active drug substance and related impurities (17)

Column: 3% Methylsilicone (OV-1) on Varaport 30 (80-100 mesh) 6 feet x 0.125 inch i.d. pyrex column

Temperature: Injector at 295°C, Detector at 300°C  
Column - Isothermal at 230°C for clioquinol and diiodo analog analysis; at 192°C isothermal for 5-chloro and 5,7-dichloro analogs and 8-hydroxyquinoline analysis.

Carrier: Helium, 40 mL/minute

Detector: Flame Ionization; H<sub>2</sub> - 30 mL/minute,  
Air - 300 mL/minute

Internal Standard: 5-Hydroxyquinoline

Sample: Derivatized with N-trimethylsilyl-imidazole in pyridine at room temperature for 15 minutes.

#### System V

Column: 1% OV-101 on Gas Chrom Q (80-100 mesh) 2 meter x 2 mm i.d. glass column (6).

Temperature: Injector at 190°C. Detector at 200°C. Column at 70°C for 3 minutes and then programmed at the rate of 8°/minute to 160°

Carrier: Nitrogen, 30 mL/minute

Detector: Flame Ionization

Internal Standard: 2,5-Dichloronitrobenzene

Sample: Derivatized with N,O-bis-(trimethylsilyl)trifluoroacetamide (BSTFA).

#### System VI

The following system has been employed for the determination of the drug in urine and blood plasma samples (18).

Column: 3% OV-17 on Gas Chrom Z (80-100 mesh) 150 cm x 0.2 cm glass column.

Temperature: Injector at 290°C. Detector at 300°C. Column at 215°C

Carrier: Nitrogen, 30 mL/minute

Detector: 1. Electron Capture ( $^{63}\text{Ni}$ ) at constant current mode  
2. Flame Ionization

Internal Standard: Chloroquinaldol(5,7-dichloro-2-methyl-8-quinolinol)

Sample: The sample was made alkaline and extracted into methylene chloride as tetrabutyl ammonium phenolate ion-pair and methylated with methyl iodide.

System VII

The following system has been employed for the determination of clioquinol in biological samples (19).

Column: 3% JXR, on Gas Chrom Q  
5 feet x 2 mm i.d. pyrex column.

Temperature: Injector at 200°C. Detector at 300°C.  
Column at 185°C

Carrier: Nitrogen, 30 mL/minute

Detector: Electron Capture ( $^{63}\text{Ni}$ , 10mCu,  
150  $\mu\text{sec}$  pulse)

Internal Standard: 5,7-Dichloro-8-hydroxyquinoline

Sample: The sample in basic solution was  
extracted as tetrahexylammonium salt  
into dichloromethane and derivatized  
to an O-methyl ether with  
iodomethane.

System VIII

The following system has been employed for the analysis of clioquinol in human plasma (7).

Column: 3% OV-17 on Gas Chrom Q (100-120  
mesh) 10 feet x 0.125 inch i.d. glass  
column.

Temperature: Injector at 230°C. Detector at 340°C.  
Column at 205°C

Carrier: Nitrogen, 50 mL/minute

Detector: Electron Capture ( $\text{Ni}^{63}$ , source  
operated with a pulsed space of  
150  $\mu\text{sec}$ )

Internal Standard: 5,7-Dichloro-8-hydroxyquinoline

Sample: Sample extracted with 1:4 methylene  
chloride:ether and derivatized with  
acetic anhydride in pyridine at 70°C.  
The derivative in ethylacetate was  
purified by a TLC procedure, extracted  
and then injected.

System IX

The following procedure has been employed for the analysis of the active ingredient and formulations for both active drug and related by-products (20).

Column: 2% SE 30 on Gas Chrom Q (100-120 mesh) 1.8 meter x  $\frac{1}{4}$  inch glass column.

Temperature: Injector at 250°C. Detector at 290°C. Column at 170°C

Carrier: Nitrogen, 45 mL/minute

Detector: Flame Ionization, Hydrogen - 36 mL/minute, Air - 300 mL/minute

Internal Standard: Aminopyrine

Sample: Derivatized in pyridine with N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA).

System X

The following procedure has been employed for the analysis of active ingredient (21, 22).

Column: 3 or 5% SE 30 on Varaport-30 (60-80 mesh)

Temperature: Column at 220°C. Injector and detector information not available.

Carrier: Nitrogen gas

Detector: Flame Ionization

Internal Standard: Hexadecane or Octadecane

Sample: Sample containing internal standard in hexane or acetonitrile derivatized with BSA at room temperature for 30 minutes

## 7.7 High Pressure Liquid Chromatography

### System I

The following system has been employed for the analysis of clinquinol and the related impurities in the active drug substance (6).

Column:	Nucleosil C <sub>18</sub> , 20 cm x 4.8 mm i.d. 10 micron column
Mobile Phase:	Acetonitrile/Water (60:40)
Flow Rate/ Temperature:	2.0 mL/minute at room temperature
Detection:	UV at 240 nm
Sample Preparation:	To a 100 mg sample dissolved in 1 mL of 50:50 mixture of triethylamine/tetrahydrofuran 1 mL of acetic anhydride is added and the solution stored at room temperature for 1 hour. A 10 µL of the sample solution is injected immediately after dilution with mobile phase.

The above system has also been employed for the analysis of active ingredient and several formulations with the following modifications: mobile phase - acetonitrile/water (80:20), UV detection at 260 nm and 1 mL/minute flow rate.

### System II

The following system has been employed for the analysis of the drug in cream formulations and in the active substance (12).

Column:	Waters, µ-Porasil (10 micron, 30 cm x 4 mm i.d.)
Mobile Phase:	Butyl chloride/water saturated butyl chloride/tetrahydrofuran/ glacial acetic acid (55:55:3:2)
Flow Rate:	Information Not Available
Temperature:	Room Temperature

Internal Standard:	Testosterone acetate
Detection	UV at 254 nm
Sample:	To 5 mL of tetrahydrofuran extract of sample containing ~1.5 mg of drug substance, 2 mL of 1:1 pyridine/ acetic anhydride is added and heated for 15 minutes at 60°C. After addition of internal standard and evaporation of solvent at 40°C, the sample is redissolved in mobile phase and injected into the column.

### System III

The following system has been employed for the analysis of clioquinol in plasma (23) and cream and ointment formulations (24).

Column:	25 cm x 2.6 mm Perkin-Elmer C <sub>18</sub> column with Brownlee a 5 cm x 40 mm C <sub>18</sub> guard column
Mobile Phase:	(1) 80% Methanol and 20% 0.05M phosphoric acid, (2) 70% Methanol and 30% 0.05M phosphoric acid
Internal Standard:	Phenylsalicylate
Flow Rate/ Temperature:	1.0 mL/minute at 40°C
Detection:	UV at 256 nm
Sample:	The plasma sample is acidified with perchloric acid and extracted with ether. The solvent is evaporated and the residue is dissolved in mobile phase before injection.

### System IV

The following high pressure liquid chromatographic procedure for the analysis of the conjugates such as glucuronide and sulfate of clioquinol in human urine has been reported (25).



Column: DuPont Zipax SAX (anion exchange)  
stainless steel column, 50 cm x 2 mm  
i.d.

Mobile Phase: Gradient from 0% of A to 25% of A at  
the rate of 3%/minute  
A. 0.02M Borate buffer with 0.1M KCl  
at pH = 9.5  
B. 0.02M Borate buffer with 0.5M  
NaClO<sub>4</sub> at pH = 9.5

Flow Rate/  
Temperature: 1.0 mL/minute at room temperature

Detection: UV at 254 nm

Sample: The urine sample is injected  
directly.

#### 7.8 Infrared Absorption Spectrophotometry (IR)

IR technique has been employed for the analysis of clioquinol in active drug and in formulations (26-28). The method based on the absorption of clioquinol in the 14.4 - 14.9 region has been shown to be specific for the active drug in the presence of several related compounds.

#### 7.9 Ultraviolet Absorption Spectrophotometry (UV)

Clioquinol has been determined quantitatively in pharmaceutical preparations (29) and in medicated feed mixes (15, 16) based on the UV absorption of the acidified solutions. The maxima at 267 nm in 3N hydrochloric acid and at 258 nm in 1.0M sulfuric acid have been employed for the quantitation of the drug.

#### 7.10 Colormetric Analysis

A colormetric assay procedure for the analysis of clioquinol and related compounds in urine samples has been described (5) in the literature. The method is based on the formation of a soluble complex with ferric ion in methyl-cellulose as solvent and absorption measurement of the complex at 650 nm. A similar method based on the formation of a complex with cupric ion at basic pH values, extraction with chloroform and measurement of the absorbance of the complex at 430 nm has also been described (30) for the analysis of pharmaceutical preparations.

### 7.11 Polarography

Clioquinol can be analyzed by polarography based on the reduction of the quinoline ring system and halide substituents. A procedure has been described (6) based on the current ( $E_{\frac{1}{2}} = -1.3V$  versus SCE) at pH~7 in 90% ethanolic solution with lithium chloride in acetate buffer as supporting electrolyte.

### 7.12 X-ray Fluorescence Analysis

Clioquinol content in active drugs has been determined by x-ray fluorescence analysis of chlorine and iodine present in the drug molecule. An indirect method based on the extraction of copper chelate and determination of copper by x-ray fluorescence has also been reported (6).

### 7.13 Gravimetric Methods

Methods for the analysis of clioquinol in active drug based on the precipitation of copper complex (31) and cadmium complex (6) in acetone have been described.

## REFERENCES

1. Bigeard, F., Clergue, H., Kerny, P. and Vaupre, R. (1964). *An. Pharm. Franc.* 22, 667-673.
2. Tanaka, H. and Tamura, Z. (1984). *J. Pharm. Sci.* 73 (11), 1647-1649.
3. Agarwal, Y. K. and Patel, D. R. (1986). *J. Pharm. Sci.* 75 (2), 190-192.
4. The Merck Index, Ninth Edition. (1976). Merck and Company. Rahway, NJ. Page 644.
5. Haskins, W. T. and Luttermoser, G. W. (1951). *Anal. Chem.* 23 (3), 456-458.
6. Personal Communication. CIBA-GEIGY Corporation.
7. Jack, D. B. and Riess, W. (1973). *J. Pharm. Sci.*, 62 (12), 1929-1932.
8. Berggren, L. and Hansson, O. (1968). *Clin. Pharmacol. Ther.* 9 (1), 67-70.
9. Liewendahl, K., Kivikangas, V. and Lamberg, B.-A. (1967). *Nucl. Med.* 6, 32-43.

10. Fischer, T., Fagurlund, C. and Hartvig, P. (1978). *Acta Dermatovener.* 58 (5)., 407-411.
11. Korzun, B. P., Brody, S. M. and Tishler, F. (1964). *J. Pharm. Sci.* 53 (8), 976-977.
12. Kubiak, E. J. and Munson, J. W. (1982). *J. Pharm. Sci.* 71 (8) 872-875.
13. Valle, R. O., Jimenez, D., Lopez, G. S. and Schroeder, I. (1978). *J. Chromatogr. Sci.* 16 (4), 162-165.
14. United States Pharmacopeia. XXI Revision. (1985). United States Pharmacopiea Convention. Rockville, MD. Page 227.
15. Cowen, T. and Heyes, W. F. (1976). *Analyst*, 101, 167-173.
16. Report from Sub-Committee on Medicinal Additives in Animal Feeds. (1981). *Analyst*. 106, 105-113.
17. Gruber, M. P., Klein, R. W., Foxx, M. E. and Campisi, J. (1972). *J. Pharm. Sci.* 61 (7), 1147-1152.
18. Hartvig, P. and Fagerlund, C. (1977). *J. Chromatogr.* 140, 170-173.
19. Degen, P. H., Schneider, W., Vuillard, P., Geiger, U. P. and Reiss, W. (1976). *J. Chromatogr.*, 117, 407-413.
20. Hartmann, Von V., and Herrmann, W. (1974). *Pharm. Ind.* 36 (3), 202-205.
21. Ohlson, B. (1973). *Sv. Farm. Tidskr.* 77 (13) 631-634); (1974). *CA.* 80, 7015u.
22. British Pharmacopiea. Volume I. (1980). London Her Majesty's Stationery Office. United Kingdon. Page 116.
23. Ezzedeen, F. W., Masoud, A. N., Stohs, S. J. and Lerman, S. J. (1981). *J. Pharm. Sci.* 70 (8), 889-891.
24. Ezzedeen, F. W., Stohs, S. J. and Masoud, A. N. (1983). *J. Pharm. Sci.* 72 (9) 1036
25. Chen, C-T., Hayakawa, K., Imanari, T. and Tamura, Z. (1975). *Chem. Pharm. Bull.*, 23 (9), 2173-2174.

26. Urbanyi, T., Sloniewsky, D. and Tishler, F. (1966). *J. Pharm. Sci.* 55 (7), 730-732.
27. Urbanyi, T. and Stober, H. (1969). *J. Pharm. Sci.* 58 (2), 232-234.
28. United States Pharmacopeia. Twentieth Revision. United States Pharmacopeia Convention. (1980). Rockville, Maryland. Pages 155-156.
29. United States Pharmacopeia. Seventeenth Revision. (1965). Mack Publishing Company. Easton, PA. Page 317.
30. Windheuser, J. J. and Chu, D. Y. (1967). *J. Pharm. Sci.* 56 (4), 519-521.
31. National Formulary. Twelfth Edition. (1965). American Pharmaceutical Association. Washington, DC. Page 208.

## CLOFAZININE

Vijay R. Kapoor

Department of Pharmaceutical Sciences,

Panjab University

Chandigarh 160014, India

1. Introduction
2. Description
  - 2.1 Name, Formula and Molecular Weight
  - 2.2 Appearance, Color and Odor
3. Physical Properties
  - 3.1 Infrared spectrum
  - 3.2 Ultraviolet and Visible Spectra
  - 3.3 Mass Spectrum
  - 3.4 Optical Rotation
  - 3.5 Melting Range
  - 3.6 Solubility
  - 3.7 Crystal Properties
4. Synthesis
5. Stability
6. Pharmacokinetics and Metabolism
  - 6.1 Pharmacokinetics
  - 6.2 Metabolism
7. Nucleic Acid Binding

### 8. Toxicity

### 9. Methods of Analysis

- 9.1 Elemental Composition
- 9.2 Identification Color Tests
- 9.3 Titrimetric Analysis
- 9.4 Radiometric Analysis
- 9.5 Spectrophotometric Analysis
  - 9.51 Colorimetric
  - 9.52 Densitometric
  - 9.53 Ultraviolet
  - 9.54 Fluorometric
- 9.6 Mass Spectrometric Analysis
- 9.7 Chromatographic Analysis
  - 9.71 Paper Chromatography
  - 9.72 Thin-Layer Chromatography
  - 9.73 Gas Chromatography
  - 9.74 High Performance Liquid Chromatography

### 10. References

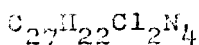
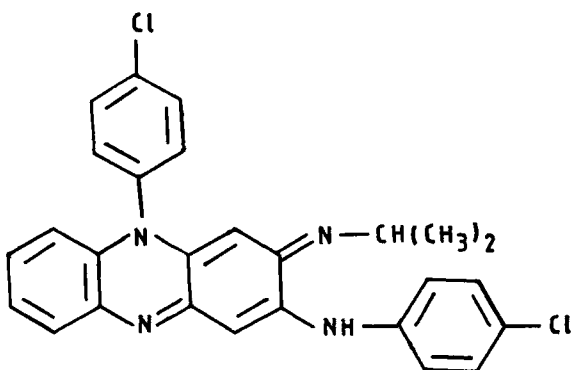
## 1. Introduction

Clofazimine (B633), a phenazine derivative, is one of the most active of a series of compounds, called rimino-compounds, synthesized by Barry and his colleagues<sup>1</sup> in suppressing experimental tuberculosis in the mouse and guinea pig. Studies have shown that clofazimine is also active against other mycobacterial infections; Mycobacterium leprae, in particular, seems to be about ten times more susceptible to clofazimine than M. tuberculosis. The efficacy of clofazimine against human leprosy, both in previously untreated patients and in patients who have relapsed with dapsone-resistant M. leprae, is well established.<sup>2-11</sup> Clofazimine is now recommended as a component of multiple-drug therapy for leprosy. The compound is also useful for treatment of chronic skin ulcers produced by M. ulcerans and it has some activity against the M. avium-intracellulare complex. The biological and pharmacological activities of clofazimine have been described.<sup>12-26</sup>

## 2. Description

### 2.1 Name, Formula and Molecular Weight

Clofazimine, also known as B663 and G30320, is 3-(4-chloroanilino)-10-(4-chlorophenyl)-2,10-dihydrophenazin-2-ylideneisopropylamine. Other



Molecular Weight: 473.4

chemical names are: 3-(4-chloroanilino)-10-(4-chlorophenyl)-2,10-dihydro-2-(isopropylimino)-phenazine; 3-(p-chloroanilino)-10-(p-chlorophenyl)-2,10-dihydro-2-(isopropylimino)phenazine; N,5-bis(4-chlorophenyl)-3,5-dihydro-3-[(1-methylethyl)imino]-2-phenazinamine. The CAS registry number is 2030-63-9. The proprietary name is Lamprene.

## 2.2 Appearance, Color and Odor

A reddish-brown, fine powder; odorless or almost odorless.

## 3. Physical Properties

### 3.1 Infrared Spectrum

The infrared spectrum (KBr disc) of clofazimine (Figure 1) exhibits principal peaks at wavenumbers 1508, 1560, 1295, 1587, 747 and 1083.<sup>27</sup>

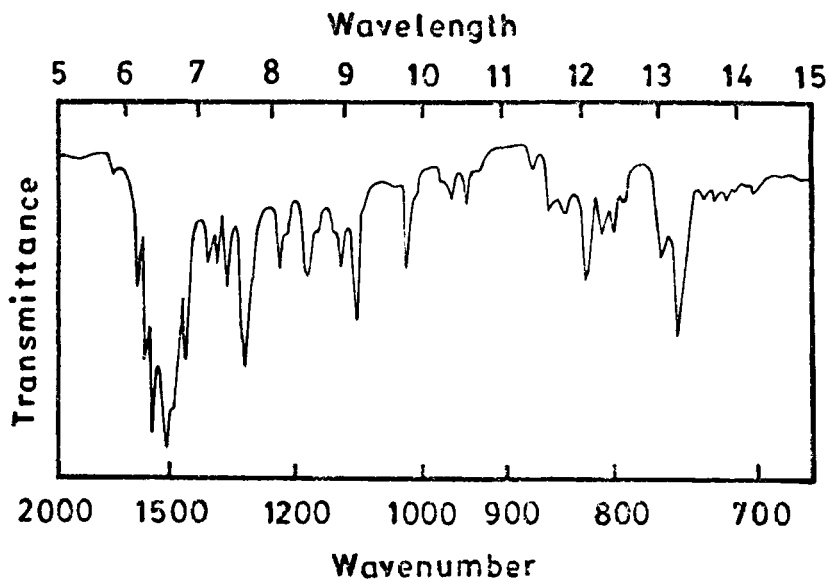


Figure 1 - Infrared spectrum of clofazimine



### 3.2 Ultraviolet and Visible Spectra

The light absorption, in the range 230 to 350 nm, of a 1-cm layer of a 0.0002% w/v solution in methyl alcohol exhibits a maximum only at 283 nm (Figure 2);<sup>27</sup> absorbance at 283 nm is about 0.3.<sup>28</sup> The light absorption in the range 230 to 600 nm of a 0.001% w/v solution in 0.01M methanolic hydrochloric acid exhibits two maxima, at 283 nm and 487 nm.<sup>29</sup> The absorbance at 283 nm is about 1.30 and at 487 nm is about 0.64.

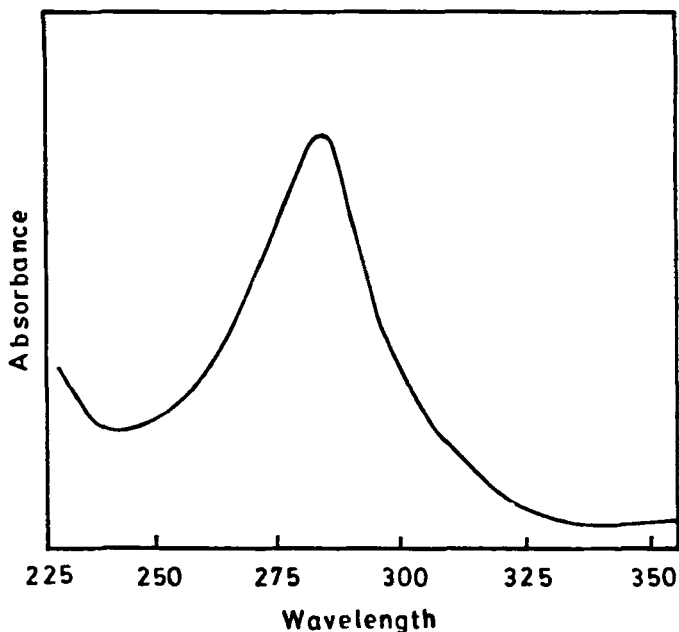


Figure 2 - Ultraviolet absorption spectrum of clofazimine

### 3.3 Mass Spectrum

The mass spectrum<sup>27</sup> of clofazimine exhibits principal peaks at  $m/z$  455, 457, 472, 474, 459, 456, 458 and 473.

### 3.4 Optical Rotation

Clofazimine exhibits no optical activity.

### 3.5 Melting Range

The melting range<sup>28</sup> of clofazimine is between 213° and 218°.

### 3.6 Solubility

Clofazimine is practically insoluble in water; soluble 1 in 700 of ethanol, 1 in 15 of chloroform, and 1 in 1000 of ether; soluble in dilute acetic acid, dimethylformamide, dioxane and macrogol 400.<sup>27,28,30</sup> A clear transparent water miscible liquid pharmaceutical vehicle for clofazimine contains Pluronic F-127.<sup>31</sup>

### 3.7 Crystal Properties

The crystal and molecular structures of two crystal forms, monoclinic and triclinic, of clofazimine have been determined from three-dimensional single-crystal counter X-ray data by using Mo K $\alpha$  radiation out to a maximum value of  $2\theta$  (Mo) = 50° on an Enraf-Nonius CAD-4 diffractometer.<sup>32</sup> The monoclinic modification of clofazimine crystallizes in the space group  $P2_1/a$  with four molecules in a cell of dimensions  $a = 7.788(14)\text{\AA}$ ,  $b = 22.960(13)\text{\AA}$ ,  $c = 13.362(7)\text{\AA}$ ,  $\beta = 98.58(12)^\circ$ ; the structure has been refined to a final value of the weighted  $R$  factor of 0.036 based on 2180 independent data with  $I > 3\sigma(I)$ . The triclinic form crystallizes in space group  $P1$  with two molecules in a cell of dimensions  $a = 10.507(4)\text{\AA}$ ,  $b = 12.852(12)\text{\AA}$ ,  $c = 9.601(2)\text{\AA}$ ,  $\alpha = 95.96(4)^\circ$ ,  $\beta = 97.22(1)^\circ$ ,  $\gamma = 89.73(6)^\circ$  and has been refined to a weighted  $R$  factor of 0.062 based on 1462 independent data with  $I = 2\sigma(I)$ . The atomic positional parameters for the monoclinic clofazimine and triclinic clofazimine are given in Table 1 and 2, respectively.

The molecular orbital calculations were performed by the CNDO/2 self-consistent field method using a local modification of the program by Pople. Molecular geometries were taken from the X-ray studies except that all C—H and N—H bond lengths were changed to 1.08 and 1.01  $\text{\AA}$ , respectively, keeping their bond directions by 50%. Table 3 gives the bond lengths in the two forms of clofazimine. The different bond angles are listed in Table 4.

Table 1- Atomic Positional Parameters for Monoclinic Clofazimine

Atom	X	Y	Z
Cl(18)	0.3867(1)	-0.07310(4)	0.39675(8)
Cl(24)	-0.3646(1)	0.44783(4)	-0.29394(8)
N(2)	-0.1755(3)	0.12955(10)	0.0017(2)
N(3)	-0.1654(3)	0.23558(10)	-0.0572(2)
N(5)	0.1451(3)	0.29139(10)	0.2623(2)
N(10)	0.2074(3)	0.17437(9)	0.3141(2)
C(1)	0.0205(4)	0.1471(1)	0.1611(2)
C(2)	-0.0829(4)	0.1628(1)	0.0674(2)
C(3)	-0.0834(4)	0.2253(1)	0.0380(2)
C(4)	-0.0044(4)	0.2651(1)	0.1033(2)
C(6)	0.2929(4)	0.3195(1)	0.4251(2)
C(7)	0.3869(4)	0.3069(1)	0.5174(2)
C(8)	0.4306(4)	0.2496(1)	0.5416(2)
C(9)	0.3733(4)	0.2055(1)	0.4749(2)
C(11)	0.2731(4)	0.2177(1)	0.3125(2)
C(12)	0.2350(4)	0.2761(1)	0.3547(2)
C(13)	0.0842(4)	0.2500(1)	0.2004(2)
C(14)	0.1025(4)	0.1876(1)	0.2247(2)
C(15)	0.2523(4)	0.1145(1)	0.3365(2)
C(16)	0.1649(4)	0.0819(1)	0.3996(2)
C(17)	0.2088(4)	0.0249(1)	0.4188(2)
C(18)	0.3372(4)	0.0003(1)	0.3740(2)
C(19)	0.4293(4)	0.0318(1)	0.3130(2)
C(20)	0.3850(4)	0.0896(1)	0.2942(2)
C(21)	-0.2045(4)	0.2874(1)	-0.1107(2)
C(22)	-0.2449(4)	0.3379(1)	-0.0635(2)
C(23)	-0.2930(4)	0.3877(1)	-0.1209(3)
C(24)	-0.2989(4)	0.3860(1)	-0.2227(2)
C(25)	-0.2590(5)	0.3365(2)	-0.2700(2)
C(26)	-0.2115(5)	0.2872(1)	-0.2139(2)
C(27)	-0.1948(4)	0.0677(1)	0.0218(2)
C(28)	-0.3328(4)	0.0592(1)	0.0886(3)
C(29)	-0.2419(5)	0.0377(1)	-0.0800(3)

Table 2- Atomic Positional Parameters for Triclinic Clofazimine

Atom	X	Y	Z
Cl(18)	0.5111(2)	0.3001(2)	0.4422(2)
Cl(24)	1.9675(3)	0.1179(3)	1.2332(3)
N(2)	1.2884(5)	0.1364(4)	0.6822(6)
N(3)	1.4514(5)	0.1463(4)	0.9018(5)
N(5)	1.0751(5)	0.3800(4)	1.1537(6)
N(10)	0.8925(5)	0.3605(4)	0.9231(6)
C(1)	1.0810(6)	0.2469(5)	0.7970(6)
C(2)	1.2271(6)	0.1933(5)	0.7880(7)
C(3)	1.3175(6)	0.2055(5)	0.9184(7)
C(4)	1.2599(6)	0.2663(5)	1.0364(6)
C(6)	0.8902(7)	0.4923(6)	1.2790(7)
C(7)	0.7546(8)	0.5483(6)	1.2909(8)
C(8)	0.6623(7)	0.5407(6)	1.1803(8)
C(9)	0.7031(7)	0.4775(6)	1.0550(8)
C(11)	0.8423(7)	0.4240(5)	1.0462(7)
C(12)	0.9371(6)	0.4304(5)	1.1583(7)
C(13)	1.1189(6)	0.3200(5)	1.0393(7)
C(14)	1.0302(6)	0.3076(5)	0.9146(7)
C(15)	0.7998(6)	0.3481(5)	0.8068(7)
C(16)	0.7528(7)	0.2623(6)	0.7996(7)
C(17)	0.6633(7)	0.2459(6)	0.6906(7)
C(18)	0.6252(6)	0.3183(6)	0.5838(7)
C(19)	0.6736(7)	0.4028(6)	0.5866(8)
C(20)	0.7598(7)	0.4201(6)	0.6996(8)
C(21)	1.5668(6)	0.1425(6)	0.9894(7)
C(22)	1.6794(7)	0.0469(6)	0.9752(8)
C(23)	1.8029(7)	0.0411(6)	1.0529(8)
C(24)	1.8113(7)	0.1265(7)	1.1417(8)
C(25)	1.7020(7)	0.2212(7)	1.1558(7)
C(26)	1.5794(6)	0.2286(6)	1.0810(7)
C(27)	1.2055(6)	0.1208(6)	0.5482(6)
C(28)A	1.1903(19)	0.2202(14)	0.4613(16)
C(29)A	1.2923(16)	0.0167(13)	0.4785(16)
C(28)B	1.1848(15)	0.0083(11)	0.5590(16)
C(29)B	1.3024(14)	0.1073(12)	0.4301(14)

Table 3- Bond Lengths ( $\text{\AA}$ ) in Clofazimine

Atoms	Monoclinic clofazimine	Triclinic clofazimine
C(1)-C(2)	1.430(3)	1.459(6)
C(1)-C(14)	1.354(3)	1.359(6)
C(2)-C(3)	1.488(3)	1.502(6)
C(2)-N(2)	1.299(3)	1.291(5)
C(3)-C(4)	1.347(3)	1.387(6)
C(3)-N(3)	1.355(3)	1.369(5)
C(4)-C(13)	1.418(3)	1.404(6)
N(5)-C(13)	1.302(3)	1.311(5)
N(5)-C(12)	1.372(3)	1.372(6)
C(6)-C(12)	1.398(3)	1.378(6)
C(6)-C(7)	1.368(3)	1.369(7)
C(7)-C(8)	1.386(3)	1.364(8)
C(8)-C(9)	1.378(3)	1.407(7)
C(9)-C(11)	1.388(3)	1.394(7)
N(10)-C(11)	1.395(3)	1.414(6)
N(10)-C(14)	1.377(2)	1.378(5)
N(10)-C(15)	1.438(3)	1.420(6)
C(11)-C(12)	1.411(3)	1.388(6)
C(13)-C(14)	1.470(3)	1.453(6)
C(27)-C(28)	1.508(4)	
C(27)-C(29)	1.521(4)	
C(27)-C(28A)		1.549(13)
C(27)-C(29A)		1.481(14)
C(27)-C(28B)		1.551(13)
C(27)-C(29B)		1.574(14)
C(27)-N(2)	1.457(3)	1.497(5)
C(21)-N(3)	1.398(3)	1.375(6)
C(18)-C1(18)	1.746(2)	1.748(6)
C(24)-C1(24)	1.744(2)	1.735(6)

Table 4 - Bond Angles (deg) in Clofazimine

Atoms	Monoclinic clofazimine	Triclinic clofazimine
C(2)-C(1)-C(14)	121.8(2)	121.9(5)
C(1)-C(2)-C(3)	117.0(2)	115.9(5)
N(2)-C(2)-C(1)	128.8(2)	128.1(4)
N(2)-C(2)-C(3)	114.1(2)	116.0(5)
C(2)-C(3)-C(4)	120.0(2)	119.7(5)
N(3)-C(3)-C(2)	113.4(2)	111.2(4)
N(3)-C(3)-C(4)	126.6(2)	129.1(5)
C(3)-C(4)-C(13)	122.7(2)	122.7(4)
C(13)-N(5)-C(12)	118.3(2)	118.2(4)
C(12)-C(6)-C(7)	122.2(3)	122.4(6)
C(6)-C(7)-C(8)	119.4(3)	118.8(6)
C(7)-C(8)-C(9)	120.1(3)	121.7(6)
C(8)-C(9)-C(11)	120.9(3)	117.5(5)
C(11)-N(10)-C(14)	121.5(2)	121.3(5)
C(11)-N(10)-C(15)	119.1(2)	119.8(5)
C(14)-N(10)-C(15)	119.4(2)	118.9(5)
N(10)-C(11)-C(9)	122.8(2)	121.3(6)
N(10)-C(11)-C(12)	117.6(2)	117.4(5)
C(9)-C(11)-C(12)	119.6(2)	121.3(6)
C(11)-C(12)-C(6)	117.8(2)	118.2(6)
C(11)-C(12)-N(5)	122.6(2)	123.2(5)
N(5)-C(12)-C(6)	119.6(2)	118.5(6)
N(5)-C(13)-C(4)	118.9(2)	118.0(5)
N(5)-C(13)-C(14)	123.8(2)	123.8(5)
C(4)-C(13)-C(14)	117.3(2)	118.2(5)
C(13)-C(14)-C(1)	120.6(2)	121.5(5)
C(13)-C(14)-N(10)	115.6(2)	116.1(5)
C(1)-C(14)-N(10)	123.8(2)	122.4(5)
N(2)-C(27)-C(28)	109.8(2)	
N(2)-C(27)-C(29)	107.1(2)	
N(2)-C(27)-C(28A)		105.5(6)
N(2)-C(27)-C(29A)		104.5(6)
N(2)-C(27)-C(28B)		104.5(6)
N(2)-C(27)-C(29B)		104.3(6)
C(28)-C(27)-C(29)	111.8(3)	
C(28A)-C(27)-C(29A)		109.4(1.1)
C(28B)-C(27)-C(29B)		109.0(1.0)
C(3)-N(3)-C(21)	131.6(2)	129.9(5)
C(2)-N(2)-C(27)	120.7(2)	119.2(4)

A view of the clofazimine molecule in the monoclinic crystals is shown in Figure 3; the molecule in the triclinic crystals is similar to this, with the exception of the disorder of the isopropyl group. As can be seen in the figure, the *p*-chlorophenyl ring at N(10) is approximately perpendicular to the dihydrophenazine plane, the torsion angles  $T_1$  defined by C(14)—N(10)—C(15)—C(16) being  $-98.8(3)^\circ$  and  $88.6(9)^\circ$  in monoclinic clofazimine and triclinic clofazimine, respectively. This approximate

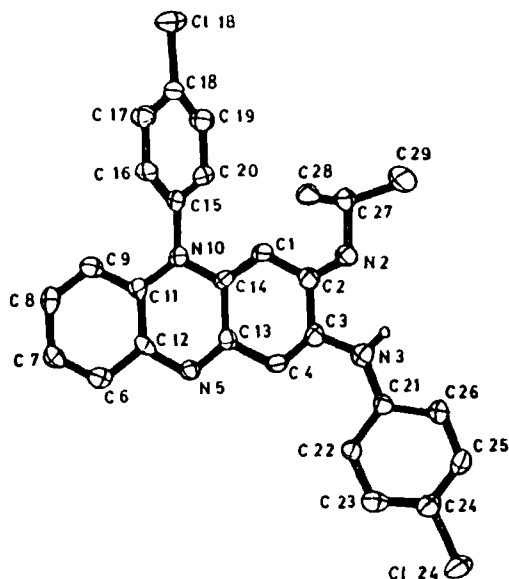


Figure 3 - View of one molecule of the monoclinic form of clofazimine. Atomic thermal ellipsoids are drawn at the 25% probability level; atom N(3) is shown as a sphere of arbitrary size, and other hydrogen atoms are omitted for clarity.

perpendicularity of the 10-(*p*-chlorophenyl) and dihydrophenazine ring system presumably stems from unfavourable interactions between the hydrogen atoms at C(1) and C(9) and those at the phenyl ortho carbon atoms C(16) and C(20) and may provide structural basis for the observation<sup>33</sup> that clofazimine does not intercalate into polynucleotides.

The crystal and molecular structure of the inactive isomeric compound isoclofazimine, 2-[(p-chlorophenyl)imino]-10-(p-chlorophenyl)-2,10-dihydro-3-(isopropylamino)phenazine, has also been described.<sup>32</sup>

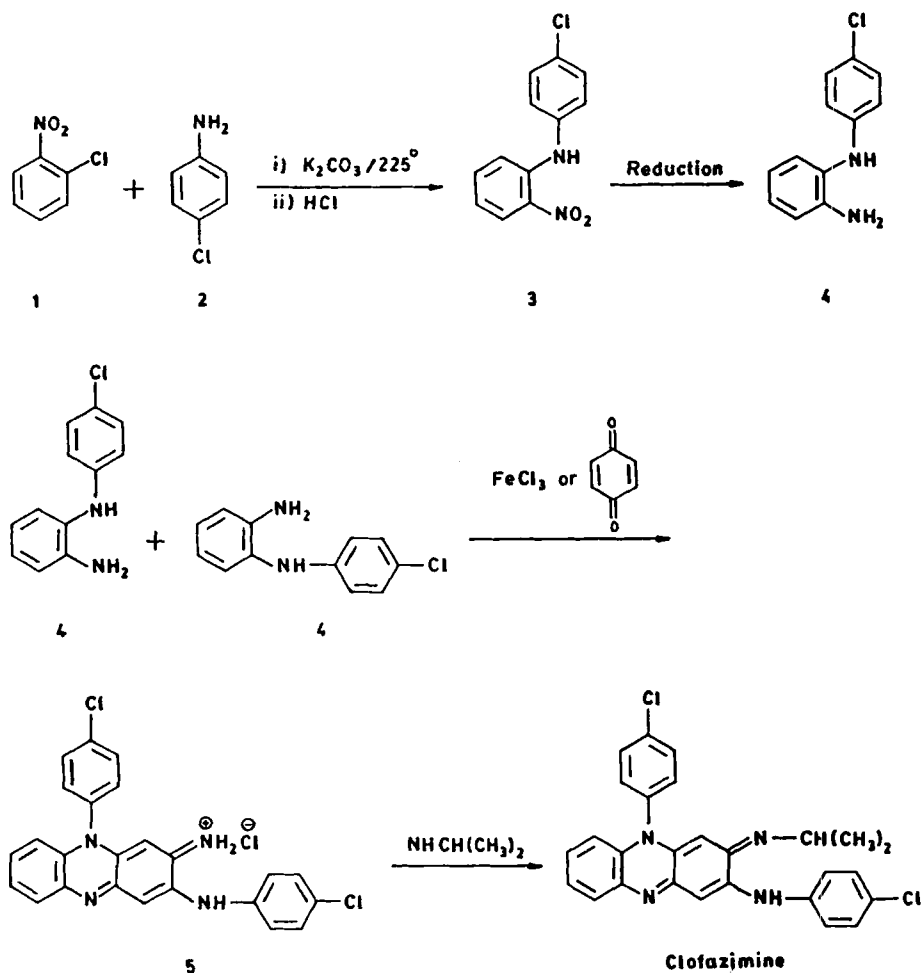
In an earlier structural study<sup>34</sup> carried on clofazimine dimethylformamide the data is: triclinic, space group  $P\bar{1}$ , with  $a = 12.435(4)\text{\AA}$ ,  $b = 12.807(5)\text{\AA}$ ,  $c = 10.424(4)\text{\AA}$ ,  $\alpha = 111.74(3)^\circ$ ,  $\beta = 112.37(3)^\circ$  and  $\gamma = 90.90(3)^\circ$ ;  $d$  (exptl) = 1.29(2) and  $d$  (calcd) = 1.293 for  $Z = 2$ ;  $R = 0.082$  for 2939 unique data.

#### 4. Synthesis

Clofazimine was synthesized by Barry and co-workers<sup>1,35-40</sup> as a result of their studies on the oxidation of N-aryl-o-phenylenediamine derivatives in the Laboratories of the Medical Research Council of Ireland, Dublin, Ireland. Scheme I outlines the synthesis of clofazimine.<sup>41</sup> It involves oxidation of N-(p-chlorophenyl)-o-phenylenediamine (4) with ferric chloride or p-benzoquinone giving the iminophenazine (5). Subsequent treatment of (5) with isopropylamine gives the "rimino" compound, clofazimine. The o-phenylenediamine (4) could be prepared<sup>42</sup> by reacting o-chloronitrobenzene (1) with p-chloroaniline (2) in presence of potassium carbonate at  $225^\circ$  followed by treatment with hydrochloric acid to give (3), which could then be reduced to (4). The isopropylamine employed in the last step could be prepared by reductive amination of acetone.<sup>43-45</sup> A method for preparing iminophenazines, in general, has been recently described.<sup>46</sup> A technique for separating clofazimine from intermediates in its synthesis by paper chromatography and on silicic acid layers has been described.<sup>47</sup>

Several analogues of clofazimine have been synthesized.<sup>48-50</sup> A recent study done on 103 clofazimine analogues active against a strain of *Mycobacterium smegmatis* 607 made resistant to the antileprosy agent has indicated that the presence of a basic nitrogen in the "rimino" side chain and a spacer distance between this nitrogen and the imino nitrogen of at least three carbon atoms are the requirements for activity.<sup>50</sup> The nitrogen may





Scheme I - Synthesis of Clofazimine

be part of an open chain or enclosed in a ring compound. Substitution elsewhere in the molecule had little effect on the activity. The analogues have been shown to have growth inhibitory activity against human-derived *Mycobacterium leprae* in murine macrophages in culture. The influence of lipophilic and steric properties on the transport of  $N^2$ -substituted phenazines to spleen of mice following oral administration has been studied.<sup>51</sup>

## 5. Stability

In general clofazimine is a stable compound. The I.P. directs that it is required to be stored in well-closed containers.<sup>28</sup>

## 6. Pharmacokinetics and Metabolism

### 6.1 Pharmacokinetics

Pharmacokinetics of clofazimine has been extensively studied.<sup>20,21,52-65</sup> Clofazimine is incompletely absorbed from the gastro-intestinal tract. Preliminary studies carried on healthy volunteers and leprosy patients suggest that less than 100% of the administered dose is absorbed and that the drug absorbed from the gastro-intestinal tract does not remain in the circulation very long, nor is it rapidly excreted, unless in the form of a metabolite.<sup>20</sup> The data show that less than 1% of the body's content of clofazimine is excreted per day. Therefore, the  $t_{1/2}$  of clofazimine in man is greater than 69 days. At this rate of excretion, one may readily calculate that the patient receiving 100 mg clofazimine will accumulate 10 g of the drug if this dosage is administered indefinitely. The findings are quite consistent with the results of studies done by Banerjee and his co-workers,<sup>21</sup> who have suggested that the low serum concentrations of clofazimine may have been due to combination of two factors: first because of incomplete absorption of the drug from the gut, and second, due to the rapid hepatic removal of absorbed drug entering the liver via the portal vein (first-pass effect). Effect of particle size and formulation on the absorption from gastro-intestinal tract has been studied by Vischer.<sup>52</sup> Only about 20% of coarsely crystalline clofazimine was absorbed from the gastro-intestinal tract; about 50% in a micronised suspension was absorbed. As a suspension in oil taken orally, absorption was 85% and from an oil-wax basis in capsules about 70%.

A recent pharmacokinetic study of clofazimine has been carried in healthy volunteers.<sup>53</sup> The pharmacokinetics were evaluated in healthy male volunteers following single and multiple oral doses

of clofazimine. Six volunteers received a single dose of 200 mg together with food. A 200-mg dose was administered in three volunteers either with or without food. In multiple-dose experiments, three volunteers were repeatedly dosed with 50 mg per day together with food for eight days. Following a single oral dose of 200 mg, the mean peak plasma concentration of clofazimine was 861 pmol/g after 8 hours. The mean terminal half-life was 10.6 days. Comparison of the bioavailability of clofazimine administered with or without food revealed a 60% higher mean area under the curve (AUC) and 30% higher mean maximum concentration ( $C_{max}$ ) value with food. The median of times to peak ( $T_{max}$ ) was 8 hours with food and 12 hours without food. In multiple dose study, good agreement was found between the mean experimental plasma concentration values and the plasma concentration profile predicted from the single-dose pharmacokinetics. The elimination half-life calculated from the terminal phase of the individual profiles after the last dose was 8.8 days. The half-life obtained from the fitted mean multiple dose profile was 10.5 days. The slow elimination of clofazimine has its implications for the treatment regimen in patients. To avoid the long lasting accumulation toward the steady state, higher daily loading doses are recommended at the beginning of the therapy followed by a daily maintenance dose. In a study done on a healthy volunteer plasma clofazimine levels following single oral doses of 200 mg and 400 mg of the drug have been determined using densitometric method (Figure 4).<sup>54</sup> A peak clofazimine concentration of 70 ng/g was reached eight hours after administration of 200 mg of clofazimine, and one of 162 ng/g four hours after the 400-mg dose. Pharmacokinetics of clofazimine in patients has also been studied.<sup>55</sup>

Barry, Conalty and their associates have studied the systemic distribution of clofazimine in the experimental animal, determining the concentration of the drug in tissue and serum.<sup>56,57</sup> Clofazimine has been shown to accumulate gradually in the tissues of experimental animals<sup>57-63</sup> with early accumulation of the drug in relatively high concentrations in the liver, lung, spleen and fat. The first study of the tissue distribution of clofazimine in man was carried out by Mansfield.<sup>64</sup>

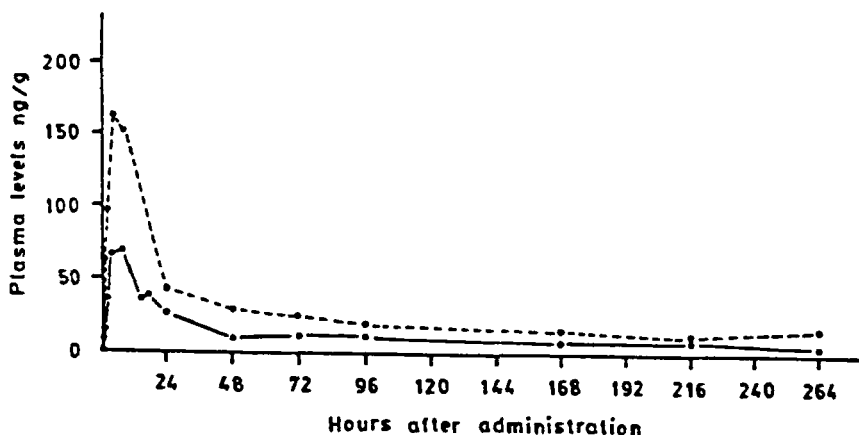


Figure 4 - Plasma levels of clofazimine in a healthy volunteer following single oral doses of 200 mg (●—●) and 400 mg (●----●) of clofazimine after on overnight fast.

Three leprosy patients were studied at autopsy; a skin biopsy was studied in a fourth patient. Tissue concentrations were analyzed by a modification of the colorimetric assay of Barry *et al.*<sup>57</sup> Tissue concentration was observed in the internal organs. Highest concentrations of clofazimine were observed in tissue with high fat content and in the bile. Tissues with a reticuloendothelial component or high vascularity also showed relatively high concentrations. However, the relatively high concentration of the drug in the kidney, which could not depend upon the presence in the organ of a large component of reticuloendothelial tissue nor of fat suggests that the accumulation of clofazimine in this organ is related to the urinary excretion of the drug. High concentration of the drug in bile and in the gall bladder is suggestive of the importance of the biliary route of elimination of clofazimine from the body.<sup>64</sup> A study carried out by Desikan and Balakrishnan<sup>65</sup> found clofazimine in all organs studied but the brain, indicating that it did not cross the blood-brain barrier.

## 6.2 Metabolism

### Metabolism of clofazimine in leprosy

patients has been investigated by Feng and co-workers.<sup>66,67</sup> Based on mass, ultraviolet and visible spectrometry, the metabolites from the urine of the patients have been characterized as: 3-(p-hydroxyanilino)-10-(p-chlorophenyl)-2,10-dihydro-2-isopropyliminophenazine (metabolite I),

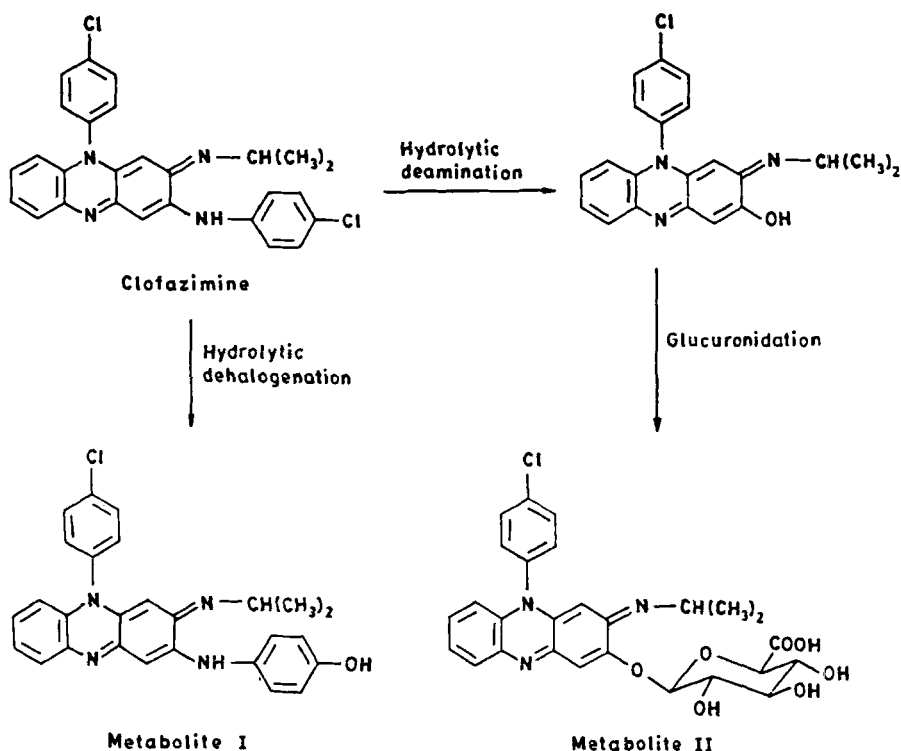


Figure 5 - Proposed pathways of the metabolite I and II formation in human.

3-(β-D-glucopyranosiduronic acid)-10-(p-chlorophenyl)-2,10-dihydro-2-isopropyliminophenazine (metabolite II), and 3-(p-chloroanilino)-10-(p-chlorophenyl)-4,10-dihydro-4(β-D-glucopyranosiduronic acid)-2-isopropyliminophenazine (metabolite III). It is suggested that metabolite I

is formed by a hydrolytic dehalogenation reaction of clofazimine and metabolite II by hydrolytic deamination followed by glucuronidation (Figure 5).<sup>66</sup> The metabolite III is proposed to be formed by an initial hydration reaction followed by glucuronidation (Figure 6).<sup>67</sup> The suggestion is based on stereochemical considerations. These three compounds are the major colored metabolites of clofazimine in human urine; however these metabolites account for less than 1% of the dose administered to these patients.

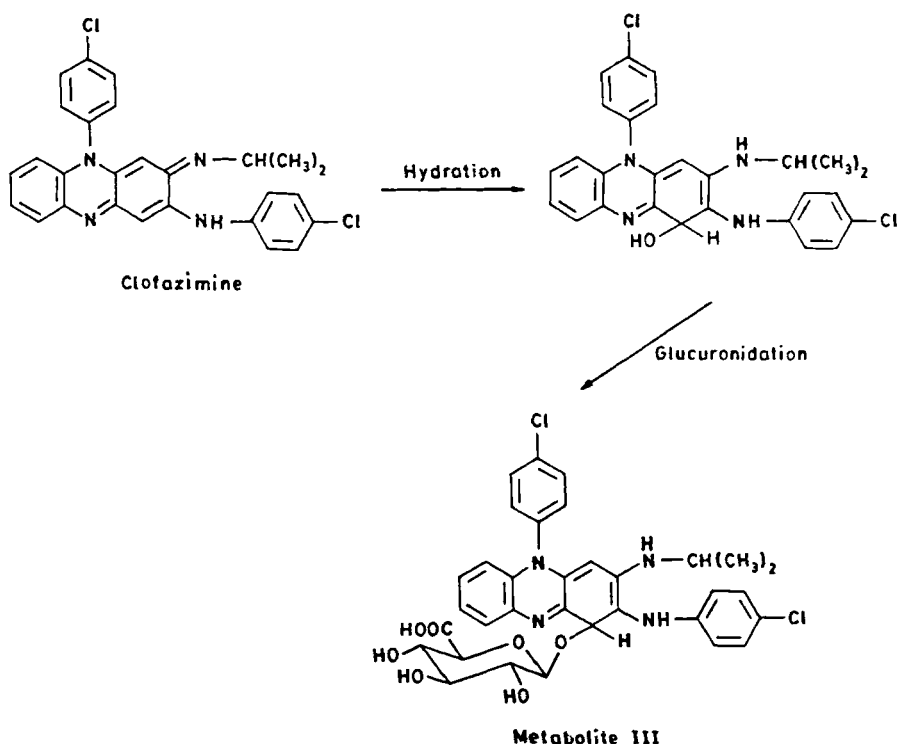


Figure 6 - Proposed pathway of the metabolite III formation in human.

There are several reports on various biochemical effects<sup>68-81</sup> of clofazimine. Effect of clofazimine on the metabolism of the other anti-loprotic drug depsone has also been reported.<sup>82</sup>

## 7. Nucleic Acid Binding

Binding studies done by Morrison and Marley<sup>33</sup> have shown that clofazimine forms stable complexes with DNA and transfer RNA. A red shift observed in the absorption spectrum of clofazimine on addition of DNA appeared specific for clofazimine binding to nucleic acid polymers. The degree of clofazimine interaction with DNA was related to guanine and cytosine content of the DNA strand. Compared with the human strand clofazimine interacted with the mycobacterial strand to give a larger red shift, consistent with the increased guanine and cytosine content of mycobacterial DNA. Clofazimine interacted with the synthetic polymucleotide, poly-guanine, whereas little interaction occurred with polyadenine, polycytosine or polyuracil. Thus, the guanine base region was a predominant site of binding to DNA. There was no evidence that clofazimine underwent intercalative binding between base pair of DNA. It is suggested that clofazimine may undergo binding along the minor groove region of DNA at appropriate base sequences which contain guanine. The resultant effect would inhibit template function of the DNA strand.

## 8. Toxicity

Stenger *et al.*<sup>83</sup> have carried out toxicity studies on clofazimine. After a single oral dose of clofazimine to mice, rats and guinea pigs the LD<sub>50</sub> has been found to be more than 4 mg/kg. The rabbit is somewhat more sensitive. Daily oral doses of 50 or 30 mg/kg, respectively over a period of six months are well tolerated by rats and monkeys. Reproduction toxicity experiments on mice, rats and rabbits have yielded no evidence for any primary embryotoxic or teratogenic action of clofazimine. In acute and chronic toxicity tests with mice, rats and rabbits clofazimine was found to be well tolerated.<sup>52</sup> A recent study<sup>84</sup> has shown that clofazimine is not mutagenic and is not an inducer of prophage  $\lambda$ , and does not eliminate plasmids from the appropriate host bacteria. Cross-resistance between clofazimine, streptomycin and rifamycin could not be demonstrated.

## 9. Methods of Analysis

### 9.1 Elemental Composition

The elemental composition of clofazimine is as follows<sup>85</sup>:

<u>Element</u>	<u>Per cent</u>
C	68.50
H	4.68
Cl	14.98
N	11.83

### 9.2 Identification Color Tests

An intense violet color is produced when 0.1 ml of hydrochloric acid is added to a solution of 2 mg of clofazimine in 3 ml of acetone. The color changes to orange-red on addition of 0.5 ml of 5M sodium hydroxide.<sup>29</sup> Application of sulfuric acid directly to the sample of clofazimine also produces a violet color.<sup>27</sup> On addition of a drop of Mandelin's reagent, which can be prepared by dissolving 0.5 g of ammonium vanadate in 1.5 ml of water and diluting to 100 ml with sulfuric acid followed by filtration through glass wool, clofazimine gives a yellow brown color.<sup>27</sup> With Marquis reagent (1 volume of formaldehyde solution and 9 volumes of sulfuric acid) clofazimine gives a violet color.<sup>27</sup>

### 9.3 Titrimetric Analysis

Clofazimine is assayed by non-aqueous titration.<sup>28,29</sup> The method involves dissolving about 0.5 g accurately weighed amount of the drug in 80 ml of acetone, and titrating with 0.1M perchloric acid in dioxane.<sup>28</sup> The end point is determined potentiometrically. A blank determination is also carried out to make any necessary correction. Each ml of 0.1M perchloric acid is equivalent to 0.04734 g of  $C_{27}H_{22}Cl_2N_4$ .

### 9.4 Radiometric Analysis

The radiometric method has been described



as a simple, rapid and quantitative test for drug susceptibility of mycobacteria. A rapid radiometric method for determining the susceptibility of Mycobacterium avium-intracellulare to eight chemotherapeutic agents including clofazimine was compared with a conventional method.<sup>86</sup> Results were available within 72 hours by radiometric testing in contrast to 21 days by conventional method. Determination of minimum inhibitory concentration of clofazimine in liquid medium by the radiometric method against Mycobacterium avium complex is reported.<sup>87</sup> Clofazimine has been employed as one of the test drugs to devise a microdilution MIC system for susceptibility testing of slowly growing mycobacteria.<sup>88</sup>

Recently, inhibition of hypoxanthine incorporation in purified suspension of Mycobacterium leprae has been demonstrated as a rapid method for in vitro screening of anti-leprosy agents.<sup>89</sup> Clofazimine significantly inhibited hypoxanthine incorporation in M. leprae. Minimum inhibitory concentration for hypoxanthine incorporation for clofazimine was found to be 100 mg/ml.<sup>89</sup>

## 9.5 Spectrophotometric Analysis

### 9.51 Colorimetric

A colorimetric method for determining clofazimine in biological fluids and tissue homogenates was originally devised by Barry et al.<sup>57</sup> The method has been successfully employed by Levy,<sup>20</sup> Banerjee et al.<sup>21</sup> and Mansfield<sup>62</sup> in the pharmacokinetic studies for the determination of clofazimine in serum, urine, feces and other tissue homogenates. The colorimetric analysis of clofazimine involves measuring the absorbance of the acid extract at 535 nm. The method has been found to be capable of measuring concentrations of clofazimine down to about 0.2 µg/ml.<sup>21</sup>

### 9.52 Densitometric

A densitometric method coupled with thin-layer chromatography has been developed by Lanyi and Dubois<sup>54</sup> to determine clofazimine in human plasma. Measurements are made in the reflectance

mode for visible absorption. A mercury light source is used with the monochromator set at  $545 \pm 15$  nm.

### 9.53 Ultraviolet

Ultraviolet spectrophotometry coupled with high performance liquid chromatography has been employed for determination of clofazimine in serum.<sup>55,90</sup> The UV spectrophotometry has also been used in the characterization of the metabolites of clofazimine.<sup>66,67</sup>

### 9.54 Fluorometric

Dill et al.<sup>91</sup> have reported a fluorometric method for analyzing clofazimine using titanous chloride and sulfuric acid. The method has been used by Levy<sup>20</sup> to determine clofazimine in plasma samples. Fluorescence was measured at 365 nm activation, 480 nm emission. Banerjee et al.<sup>21</sup> have also used fluorometry to determine clofazimine in urine and tissue homogenates.

## 9.6 Mass Spectrometric Analysis

Mass spectrometric analysis has been used in the identification of clofazimine in mouse spleen macrophages.<sup>63</sup> Characterization of the metabolites of clofazimine has been achieved using mass spectrometry as the major technique.<sup>66,67</sup>

## 9.7 Chromatographic Analysis

### 9.71 Paper Chromatography

Paper chromatography has been used to separate clofazimine from intermediates in its synthesis.<sup>47</sup>

### 9.72 Thin-Layer Chromatography

The following thin-layer chromatographic systems have been recommended for the identification of clofazimine:

Solvent System	Plate	R <sub>f</sub>	Reference
Methanol-Strong ammonia solution (100:1.5)	Silica gel G, 250 $\mu$ m thick, dipped in, or sprayed with, 0.1M KOH in methanol, and dried	0.70	27
Cyclohexane-Toluene-Diethylamine (75:15:10)	Same as above	0.57	27
Chloroform-Methanol (90:10)	Same as above	0.59	27
Ethyl acetate-Benzene (10:90)	Silica gel type H plates containing 0.1M KOH (two elutions)	0.39	63
1-Butanol-Benzene-Water-Methanol (2:1:1:1.25)	Precoated TLC plates, 250- $\mu$ silica gel GHLF	0.65	66,67
Toluene-Acetic acid-water (50:50:4)	Precoated HPTC Silica gel 60 plates (20 x 10 cm), pre-developed in chloroform-methanol (1:1) prior to use	0.36	54

Detection of spot of clofazimine on the plate has been carried out by acidified iodoplatinate solution<sup>27</sup> or visualization by color and UV absorption.<sup>66</sup>

### 9.73 Gas Chromatography

Clofazimine was not eluted from the gas chromatography system: column, 2.5% SE-30 on 80-100 mesh Chromosorb G (acid-washed and dimethyl-dichlorosilane-treated), 2m x 4 mm internal diameter glass column; column temperature, between

100 and 300°; carrier gas, nitrogen at 45 ml/min.<sup>27</sup>

### 9.74 High Performance Liquid Chromatography

Peters et al.<sup>55</sup> have reported a high performance liquid chromatography method to determine clofazimine in human or rat blood plasma. After addition of 10 ml aliquots of blood plasma containing clofazimine, extraction with chloroform-methanol (4:1), centrifugation, evaporation of the separated organic layer and reconstitution of the residue in 150 µl mobile phase solution (0.0425M phosphoric acid in 81% methanol) and 0.5 ml hexane, clofazimine-containing samples were chromatographed on a reversed-phase Ultrasphere-octyl column at 40°. Clofazimine was detected at 285 nm; its retention time was 9.6 minute. The lower limit of sensitivity was established to be 10 ng/ml. Gidoh et al.<sup>55</sup> have published a HPLC method for the simultaneous analysis of clofazimine, dapsone and rifampicin and their metabolites in serum using a µBondapak C<sub>18</sub> column. HPLC has also been employed in the investigations on the metabolism of clofazimine.<sup>66,67</sup>

### 10. References

1. V.C. Barry, J.G. Belton, M.L. Conalty, D.M. Denney, D.W. Edward, J.F. O'Sullivan, D. Twomey and F. Winder, *Nature*, 179, 1013 (1957).
2. J.H.S. Pettit and R.J.W. Rees, *Int. J. Lepr.*, 34, 391 (1966).
3. J.H.S. Pettit, R.J.W. Rees and D.S. Ridley, *Int. J. Lepr.*, 35, 25 (1967).
4. Y.T. Chang, *Int. J. Lepr.*, 35, 78 (1967).
5. J.M. Gangas, *Lepr. Rev.*, 38, 225 (1967).
6. A.G. Warren, *Lepr. Rev.*, 39, 61 (1968).
7. F.M. Imkamp, *Lepr. Rev.*, 39, 119 (1968).
8. J. Languillon, *Med. Trop. (Marseilles)*, 29, 497 (1969); C.A., 72, 41611e (1970).

9. L. Levy, C.C. Shepard and P. Fasal. *Am. J. Trop. Med. Hyg.*, 21, 315 (1972).
10. R.R. Jacobson, *Int. J. Lepr.*, 49, 510 (1981).
11. S.G. Browne, D.J. Harman, H. Waudby and A.C. McDougall, *Int. J. Lepr. Other Mycobact. Dis.*, 49, 167 (1981).
12. B.K. Acharya, J.M. Robson and F.M. Sullivan, *Am. Rev. Respirat. Diseases*, 80, 871 (1959).
13. W. Steenken, Jr., V. Montalbino and M.M. Smith, *Am. Rev. Respirat. Diseases*, 81, 764 (1960).
14. F. Grumbach and S. Clavel, *Ann. Inst. Pasteur*, 99, 567 (1960); *C.A.*, 55, 9645f (1961).
15. N.J.L. Gilmour, *Brit Vet. J.*, 122, 517 (1966).
16. W.A. Vischer, *Arzneim. Forsch*, 18, 1529 (1968).
17. W.A. Vischer, *Arzneim. Forsch*, 20, 714 (1970).
18. C.C. Shepard, L.L. Walker, R.M. Van Landingham and M.A. Redus, *Proc. Soc. Exp. Biol. Med.*, 137, 725 (1971).
19. C.C. Shepard, L.L. Walker, R.M. Van Landingham and M.A. Redus, *Proc. Soc. exp. Biol. Med.*, 137, 728 (1971).
20. L. Levy, *Am. J. Trop. Med. Hyg.*, 23, 1097 (1974).
21. D.K. Banerjee, G.A. Ellard, P.T. Gammon and M.F.R. Waters, *Am. J. Trop. Med. Hyg.*, 23, 1110 (1974).
22. P. Sensi and G. Gialdroni-Grassi, in *Burger's Medicinal Chemistry*, Fourth Edn. Part II, M.E. Wolff, Ed., John Wiley & Sons, New York, 1979, p. 308.
23. G.L. Mandell and M.A. Sande, in *Goodman and Gilman's The Pharmacological Basis of Therapeutics*, Seventh Edn., A. Goodman Gilman, L.S. Goodman, T.W. Rall and F. Murad, Eds., Macmillan Publishing Co., New York, 1985, p. 1213.

24. V. Ausina, M.J. Condom, B. Mirelis, M. Luquin, P. Coll and G. Prats, *Antimicrob. Agents Chemother.*, 29, 951 (1986).
25. M. Luquin, B. Mirelis, V. Ausina, M.J. Condom, L. Matas, *Int. Congr. Ser. Excerpta Med.*, 697, 188 (1986); C.A., 105, 222536e (1986).
26. D.M. Yajko, P.S. Nassos and W.K. Hadley, *Antimicrob. Agents Chemother.*, 31, 117 (1987).
27. Clarke's Isolation and Identification of Drugs, Second Edn., A.C. Moffat (Sr. Con. Ed.), The Pharmaceutical Press, London, 1986, p. 476.
28. *Pharmacopoeia of India 1985*, Vol. I, Controller of Publications, Delhi, 1985, p. 127.
29. *British Pharmacopoeia 1988*, Vol. I, Her Majesty's Stationary Office, London, 1988, p. 145.
30. Martindale The Extra Pharmacopoeia, Twenty-eighth Edn., J.E.F. Reynolds, Ed., The Pharmaceutical Press, London, 1982, p. 1488.
31. J.Z. Krezanoski, French Patent 2,400,930; C.A., 91, 181465c (1979).
32. U. Rychlewska, M.B.H. Broom, D.S. Eggleston and D.J. Hodgson, *J. Am. Chem. Soc.*, 107, 4768 (1985).
33. N.E. Morrison and G.M. Marley, *Int. J. Lepr. Other Mycobact. Dis.*, 44, 475 (1976).
34. D.S. Eggleston, W.E. Marsh, D.J. Hodgson, *Acta Crystallogr., Sect. C*, 040, 288 (1984).
35. V.C. Barry, J.G. Belton, M.L. Conalty and D. Twomey, *Nature*, 162, 622 (1948).
36. V.C. Barry, J.G. Belton, J.F. Chambers, M.L. Conalty, R. Kelly and D. Twomey, *Proc. Roy. Irish Acad.*, 55B, 157 (1953).
37. V.C. Barry, J.G. Belton, J.F. O'Sullivan and D. Twomey, *J. Chem. Soc.*, 888 (1956).

38. V.C. Barry, J.G. Belton, J.F. O'Sullivan and D. Twomey, *J. Chem. Soc.*, 3347 (1956).
39. V.C. Barry, M.L. Conalty and E.E. Gaffney, *J. Pharm. Pharmacol.*, 8, 1089 (1956).
40. V.C. Barry, J.G. Belton, J.F. O'Sullivan and D. Twomey, *J. Chem. Soc.*, 859 (1958).
41. *Profiles in Drug Syntheses*, Vol. I, V.N. Gogte, Ed., Gokul Publishers, Bombay, 1982.
42. G.M. Barnett, P.C. Crofts and D.R. Hey, *J. Chem. Soc.*, 227 (1949).
43. A. Skita and F. Keil, *Ber.*, 61, 1682 (1928).
44. C.F. Winans and R. Adkins, *J. Am. Chem. Soc.*, 55, 2051 (1933).
45. D.G. Norton, V.E. Haury, F.C. Davis, L.J. Mitchell and S.A. Ballard, *J. Org. Chem.*, 19, 1054 (1954).
46. J.F. O'Sullivan, *J. Chem. Res.*, 52 (1984).
47. E. Ragazzi, *Boll. Chim. Farm.*, 100, 402 (1961); *C.A.*, 56, 10283e (1962).
48. L. Levy, *Lepr. Rev.*, 52, 23 (1981).
49. D. Twomey, *J. Heterocycl. Chem.*, 23, 615 (1986).
50. J.F. O'Sullivan, M.L. Conalty and N.E. Morrison, *J. Med. Chem.*, 31, 567 (1988).
51. E.B. Canavan, A.G. Esmonde, J.F.P. Freely, J.M. Quigley and R.F. Timoney, *Eur. J. Med. Chem.-Chim. Ther.*, 21, 199 (1986).
52. W.A. Vischer, *Lepr. Rev.*, 40, 107 (1969).
53. Z. Schaad-Lanyi, W. Dieterle, J.P. Dubois, W. Theobald and W. Vischer, *Int. J. Lepr. Other Mycobact. Dis.*, 55, 9 (1987).
54. Z. Lanyi and J.P. Dubois, *J. Chromatogr.*, 232, 219 (1982).

55. J.N. Peters, K.J. Hamme and G.R. Gordon, *J. Chromatogr.*, 229, 503 (1982).
56. V.C. Barry and M.L. Conalty, *Lepr. Rev.*, 36, 3 (1965).
57. V.C. Barry, K. Baggie, J. Byrne, M.L. Conalty and F. Winder, *Irish J. Med. Sci.*, 416, 345 (1960).
58. V.C. Barry and M.L. Conalty, *Am. Rev. Tuberc. Pulmonary Diseases*, 78, 62 (1958).
59. M.L. Conalty and R.D. Jackson, *Brit. J. Exptl. Pathol.*, 43, 650 (1962).
60. M.L. Conalty and R.D. Jackson, *Ann. Histochimie*, 7 (suppl.), 221 (1962).
61. R.O. Drabkina and T.S. Gizburg, *Rozhledy Tuberk.*, 22, 425 (1962); *C.A.*, 57, 15742e (1962).
62. M.L. Conalty, *Irish J. Med. Sci.*, 49, 497 (1966).
63. R.T. Alpin and A.C. McDoughall, *Experientia*, 31, 468 (1975).
64. R.F. Mansfield, *Am. J. Trop. Med. Hyg.*, 23, 1116 (1974).
65. K.V. Desikan and S. Balakrishnan, *Lepr. Rev.*, 47, 107 (1976).
66. P.C.C. Feng, C.C. Fenselau and R.R. Jacobson, *Drug Metab. Dispos.*, 9, 521 (1981).
67. P.C.C. Feng, C.C. Fenselau and R.R. Jacobson, *Drug Metab. Dispos.*, 10, 286 (1982).
68. H. Mathies and U. Röss, *Arzneim. Forsch.*, 20, 183 (1970).
69. M.J. Claine, *Infect. Immunity*, 2, 601 (1970); *C.A.*, 74, 11664y (1971).
70. M.J. Claine, *Role Lymphocytes Macrophages Immunol. Response*, 13th Int. Congr. Haematol., 43 (1970), D.C. Danonde, Ed., Springer, Berlin; *C.A.*, 75, 128049p (1971).



71. K. Lavrijssen, P. Lavrijssen and R. Lontie, *Biochem. Pharmacol.*, 26, 1345 (1977).
72. M. Gidoh, S. Tsutsumi, M. Narita and K. Fukushi, *Nippon Rai Gakkai Zasshi*, 48, 159 (1979); *C.A.*, 93, 215381f (1980).
73. T. Moises and C.M. Finlay, *Rev. Cienc. Biol. (Havana)*, 14, 49 (1983); *C.A.*, 101, 122644n (1984).
74. Y. Niwa, T. Sakane, Y. Miyachi and M. Ozaki, *J. Clin. Microbiol.*, 20, 837 (1984).
75. J. Sarracent and C.M. Finlay, *Int. J. Lepr. Other Mycobact. Dis.*, 52, 154 (1984).
76. R. Anderson, *Antimicrob. Agents Chemother.*, 27, 257 (1985).
77. A.J. Van Rensburg and R. Anderson, *Recent Adv. Chemother., Proc. 14th Int. Congr. Chemother.*, 799 (1985), J. Ishigami, Ed., University of Tokyo Press, Tokyo, Japan; *C.A.*, 105, 218377m (1986).
78. R. Anderson, P. Lukey, C. Van Rensburg and U. Dippenaar, *Int. J. Immunopharmacol.*, 8, 605 (1986).
79. B.M. Zeis and R. Anderson, *Int. J. Immunopharmacol.*, 8, 731 (1986).
80. A.A. Wadee, R. Anderson and A.R. Rabson, *J. Antimicrob. Chemother.*, 21, 65 (1988).
81. A. Sahu, K. Saha, A. Kashyap and A. K. Chakrabarty, *Immunopharmacology*, 15, 143 (1988).
82. J. George, S. Balakrishnan and V.N. Bhatia, *Indian J. Med. Res.*, 87, 151 (1988).
83. E.G. Stenger, L. Aepli, E. Peheim and P.E. Thomann, *Arzneim. Forsch.*, 20, 794 (1970).
84. H.L. David, N. Rastogi, S. Clavel-Seres and F. Clement, *Zentralbl. Bakteriол., Mikrobiol. Hyg., Ser A*, 226, 292 (1987); *C.A.*, 108, 3324t (1988).

85. Merck Index, 10th Edn., M. Windholtz, Ed., Merck & Co., Inc., Rahway, N.J., U.S.A., 1983, p.337.
86. B.G. Yangco, E.A. Eikman, D.A. Solomon, S.C. Deresinski and J.A. Madden, Antimicrob. Agents Chemother., 19, 534 (1981).
87. L.B. Heifets, M.D. Iseman and P.J. Lindholm-Levy, Drugs Exp. Clin. Res., 13, 529 (1987); C.A., 108, 34701t (1988).
88. R.J. Wallace, Jr., D.R. Nash, L.C. Steele and V. Steingrube, J. Clin. Microbiol., 24, 976 (1986).
89. P.R. Wheeler, J. Med. Microbiol., 25, 167 (1988).
90. M. Gidoh, S. Tsutsumi and S. Takitani, J. Chromatogr., 223, 379 (1981).
91. W.A. Dill, L. Chucot and A. J. Glazko, Int. J. Lepr., 38, 355 (1970).

## ETOPOSIDE

Joost J.M. Holthuis, J. Jantina Kettenes-van den Bosch,  
and Auke Bult

University of Utrecht  
Department of Pharmaceutical Analysis  
Utrecht, The Netherlands.

1. History
2. Description
  - 2.1. Nomenclature, Formula, and Molecular Weight
  - 2.2. Appearance, Odour, and Colour
3. Synthesis
4. Physical Properties
  - 4.1. Ultraviolet Spectrum
  - 4.2. Infrared Spectrum
  - 4.3. Fluorescence Emission Spectrum
  - 4.4. Nuclear Magnetic Resonance Spectrum
  - 4.5. Mass Spectrum
  - 4.6. Melting Range
  - 4.7. Differential Scanning Calorimetry
  - 4.8. Optical Rotation
  - 4.9. Dissociation Constant
  - 4.10. Electrochemistry
5. Methods of Analysis
  - 5.1. Thin Layer Chromatography
  - 5.2. High Performance Liquid Chromatography
6. Stability and Degradation
  - 6.1. Stability in Aqueous Solutions
  - 6.2. Stability in Plasma and Urine
7. Pharmacology
  - 7.1. Mechanism of Action
  - 7.2. Pharmacokinetics
  - 7.3. Clinical Activity
  - 7.4. Clinical Toxicity

8. Analysis of Etoposide and Metabolites in Biological Matrices
  - 8.1. Etoposide
  - 8.2. Etoposide Metabolites
9. References

## 1. HISTORY

Etoposide is a semi-synthetic derivative of epipodophyllotoxin. It is used for the treatment of lung cancer, testicular cancer, lymphoma and several types of leukaemia, and is one of the most active drugs against small cell lung cancer [1]. Etoposide was synthesized from podophyllotoxin in 1963, in the Sandoz Laboratories.

Podophyllotoxin is isolated from the dried roots and rhizomes of species of the genus *Podophyllin*. The medicinal properties of the ethanolic extracts of these roots and rhizomes (podophyllin) have been known for more than 150 years.

Podophyllin was used as a purgative, anthelmintic, choleretic, and vesicant agent. Common sources of podophyllin are the may apple or American mandrake (*Podophyllin peltatum* L.) and *Podophyllum emodi* Wall. [2]. Podophyllin contains several podophyllotoxin derivatives among which podophyllotoxin proved to be the most active cytotoxic compound.

Several podophyllin components possess considerable anti-tumour activity but are considered to be unacceptable for use in humans because of their severe side effects: their toxicity prevents administration of doses high enough to give sufficient therapeutic effect. In an attempt to find compounds with an acceptable therapeutic index, a variety of derivatives were synthesized from natural podophyllotoxin [3-5]. Etoposide proved to be one of the most promising compounds.

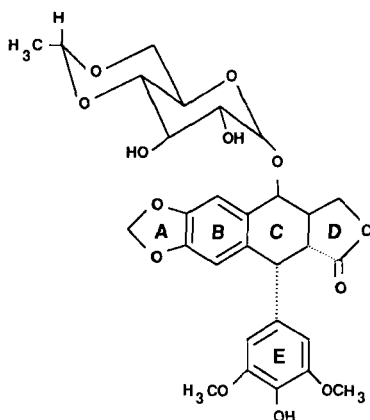
## 2. DESCRIPTION

### 2.1. Nomenclature, Formula, and Molecular Weight

The generic name is etoposide (33419-42-0). Other names are VP 16-213, VP 16, EPEG, NSC 141540. The trade name of the drug is Vepesid®.

The Chemical Abstracts' name is 4'-O-demethyl-1-O-(4,6-O-ethylidene-β-D-glucopyranosyl)epipodophyllotoxin (IUPAC) or 5-(4,6-O-ethylidene-β-D-glucopyranosyloxy)-2,5,5a,6,8a,9-hexahydro-9-(4-hydroxy-3,5-dimethoxyphenyl)furo[3',4':6,7]-naphtho[2,3-d] [1,3] dioxo-8-one.

The molecular formula of etoposide is  $C_{29}H_{32}O_{13}$ ; its molecular weight 588.6.



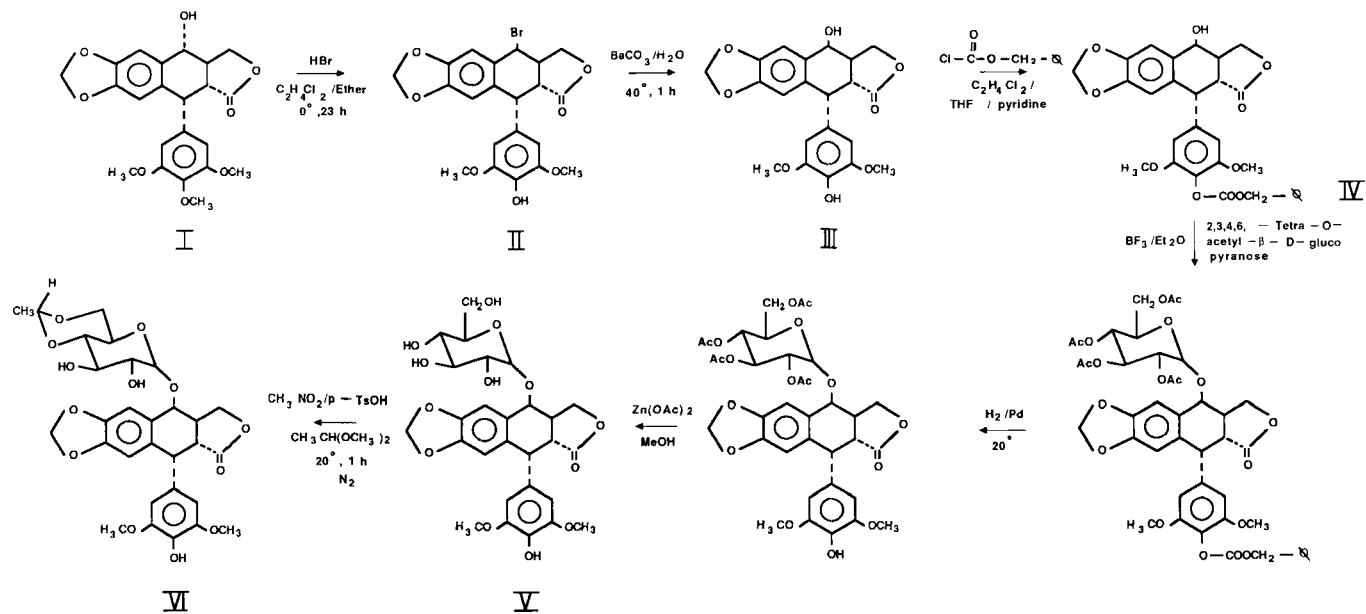
## 2.2. Appearance, Odour, and Colour

Etoposide is a white, odourless and amorphous powder.

## 3. SYNTHESIS OF ETOPOSIDE

The synthesis of etoposide from naturally occurring podophyllotoxin I (Scheme 1) is described in ref. [3-5]. Podophyllotoxin is treated with HBr in 1,2-dichloroethane, resulting in 1-bromo-1-desoxyepipodophyllotoxin, which demethylates to 1-bromo-4'-demethylepipodophyllotoxin (II) when the reaction mixture is kept at 0°C for about 24 hours. By treatment of II with BaCO<sub>3</sub> in an acetone/water mixture, the bromine is replaced by a hydroxyl group, resulting in 4'-demethylepipodophyllotoxin (III). After protection of the phenolic hydroxyl with benzyl chloroformate, the 4'-OH group is coupled with 2,3,4,6-tetra-O-acetyl-β-D-glucopyranose. The sugar moiety probably enters from the less hindered side, because glycosidation of podophyllotoxin itself also results in an epi product [4].

The protecting group at the 4'-OH is removed by hydrogenolysis with H<sub>2</sub>/Pd and the acyl groups by hydrolysis with Zn(OAc)<sub>2</sub> in methanol. During the hydrolysis, about 30% of the compound is converted into a mixture of the hydroxy acid (by opening of the lactone ring) and the *cis* lactone. These products are easily removed by crystallization. The last step in the synthesis is the reaction with



Scheme 1. Synthesis of etoposide.

acetaldehyde dimethyl acetal in nitromethane, with *p*-toluenesulfonic acid as a catalyst. Of the 0-4,6 cyclic acetals, the isomer with the equatorial methyl group predominates. Minor quantities of the axial isomer are eliminated in the purification procedure [5].

#### 4. PHYSICAL PROPERTIES

##### 4.1. Ultraviolet Spectrum

The ultraviolet spectrum of a 92  $\mu\text{M}$  solution of etoposide in absolute methanol (Figure 1) shows an absorption maximum

at 283 nm. The specific extinction ( $E_{1\text{cm}}^{1\%}$ ) at 283 nm is 72.2

( $\epsilon = 4245 \text{ l.mol}^{-1}.\text{cm}^{-1}$ ) [6]. The ultraviolet spectrum was recorded with a double beam Shimadzu Spectrophotometer UV-200 in a 1 cm silica cell.

##### 4.2. Infrared Spectrum

The IR spectrum of etoposide (KBr tablet) is shown in Figure 2. The spectrum was recorded with a Jouan-Jasco IRA-1 grating infrared spectrometer.

Characteristic bands are the carbonyl stretch vibration of the strained *trans* lactone ring at  $1775 \text{ cm}^{-1}$ , the OH stretch vibration of the phenolic and sugar OH groups at  $3400 \text{ cm}^{-1}$ , the aromatic band at  $1610$ ,  $1515$  and  $1485 \text{ cm}^{-1}$ , and the C-O stretch vibration at  $1250 \text{ cm}^{-1}$ .

##### 4.3. Fluorescence Spectrum

The fluorescence emission spectrum of etoposide (Figure 3) was recorded with a Kontron SFM 25 fluorimeter. An excitation wavelength of 295 nm and a scan rate of 60 nm/min were used.



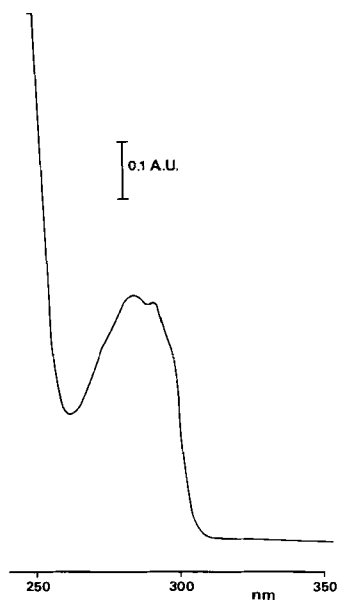


Figure 1. The ultraviolet spectrum of etoposide (92 mM) in methanol.

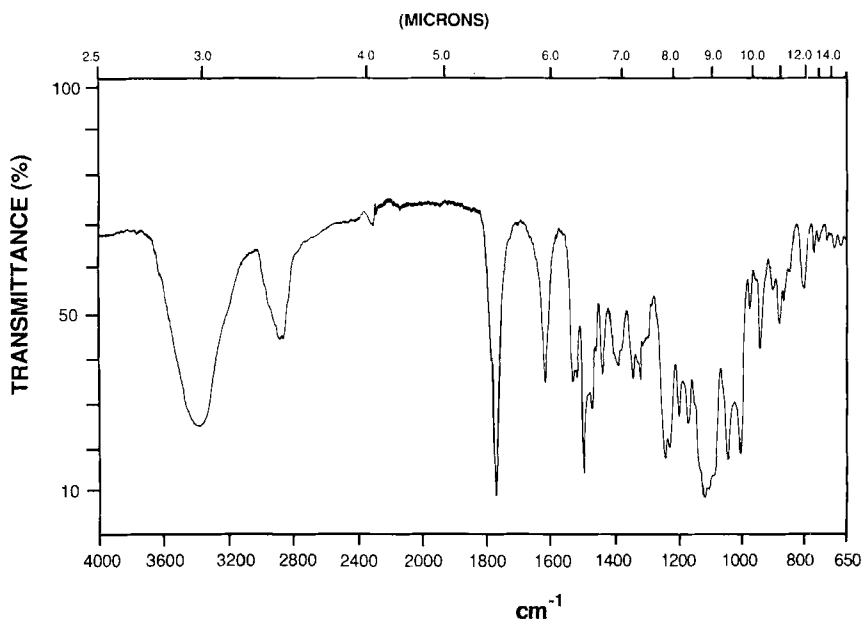


Figure 2. The infrared spectrum of etoposide.

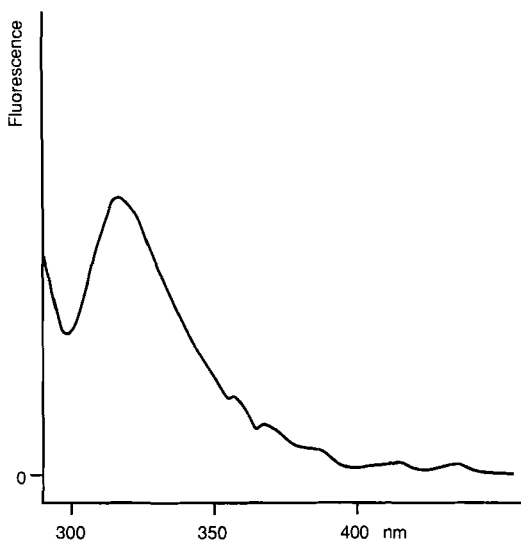


Figure 3. The emission spectrum (not-corrected) of etoposide (9.4  $\mu$ M) in methanol.

#### 4.4. Nuclear Magnetic Resonance Spectrum

The proton NMR was recorded in deuteriochloroform containing a drop of dimethyl sulfoxide- $d_6$ , with a Bruker AM-500 spectrometer at a frequency of 500.14 MHz. The internal standard was DMSO (at 2.49 p.p.m.). The spectrum between 2.70 and 5.00 p.p.m. is reproduced in Figure 4.

Chemical shift assignments (Table I) and coupling constants for ring C and D protons and for the glucose moiety (Table II) were made on the basis of proton double irradiation experiments and integrated intensity measurements. They are in agreement with those reported by Strife and Jardine [7,8].

The natural abundance  $^{13}\text{C}$  NMR spectrum was recorded with a Bruker SP-200 WB instrument at a frequency of 50.3 MHz, with deuteriochloroform containing a drop of dimethyl sulfoxide- $d_6$  as the solvent. DMSO (at 39.5 p.p.m.) was used as the internal standard. The proton-noise decoupled spectrum is reproduced in Figure 5; the spectral assignments are presented in Table III.

Since some of the chemical shift values differ only slightly, the assignments for the corresponding signals may be interchanged.

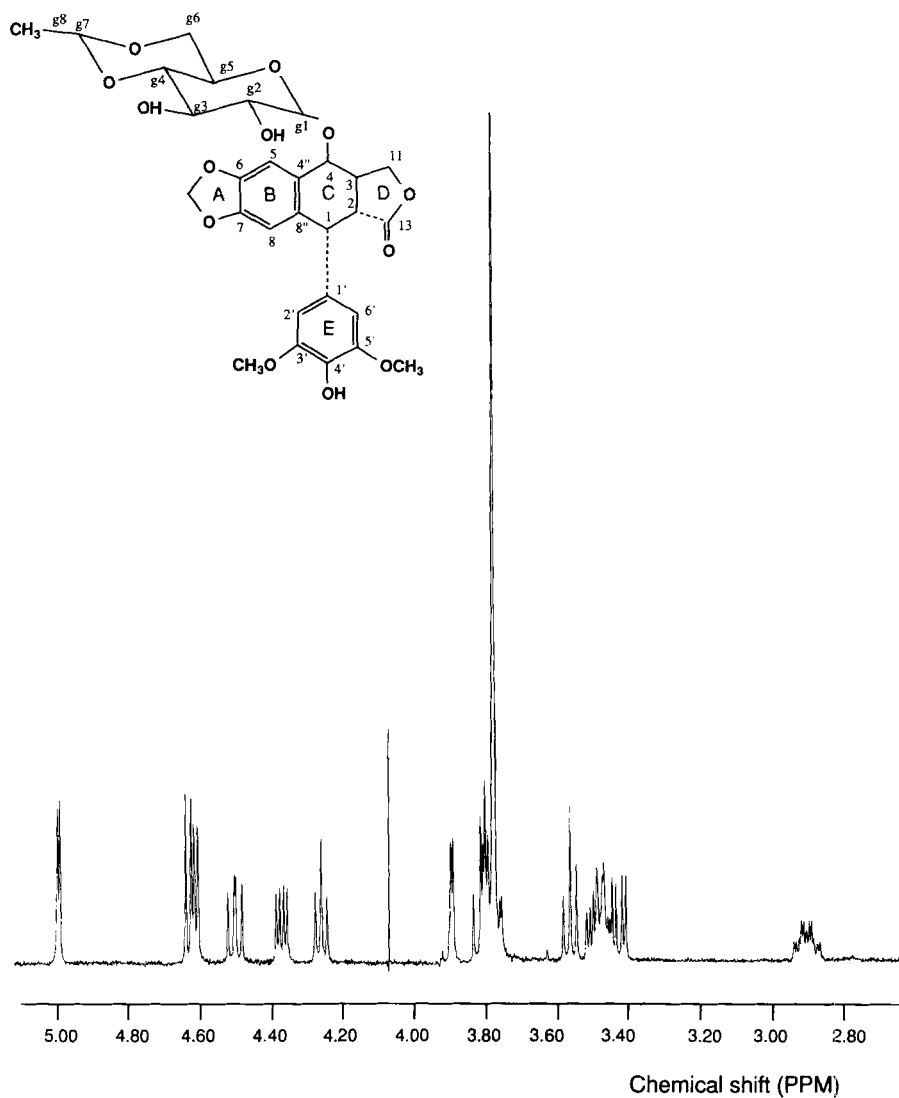


Figure 4. Proton NMR spectrum (2.70-5.10 p.p.m.) of etoposide.

Table I.  $^1\text{H}$  NMR assignments for etoposide in deuteriochloroform.

chemical shift $\delta$ (p.p.m.)	multiplicity	number of protons	assignment (protons at carbon number)
1.38	d	3	g8
2.88	m	1	3
3.32	m	2	g4, g5
3.38	dd	2	2, g2
3.58	t	1	g6a
3.67	t	1	g3
3.72	s(br)	1	g3-OH
3.75	s	6	$\text{OCH}_3$
4.05	s(br)	1	g2-OH
4.18	dd	1	g6e
4.21	t	1	11'
4.45	dd	1	11
4.55	d	1	g1
4.57	d	1	1
4.75	q	1	g7
4.94	d	1	4
5.68	s	1	4'-OH
5.98	d,d (AB)	2	A
6.25	s	2	2',6'
6.52	s	1	8
6.84	s	1	5

Table II. Proton-proton coupling constants for etoposide.

coupled protons	$J(\text{Hz})$
1,2	4.9
2,3	14.0
3,4	3.4
3,11	10.5
3,11'	8.5
11,11'	8.5
A,A	$\sim 1$
g1,g2	7.9
g2,g3	7.9
g3,g4	7.9
g4,g5	
g5,g6a	9.6
g5,g6e	4.3
g6a,g6e	9.6
g7,g8	5.0

Table III.  $^{13}\text{C}$  NMR assignments for etoposide.

chemical shift $\delta$ (p.p.m.)	assignment (carbon number)
19.9	g8
37.2	1
40.6	3
43.2	2
55.9	$\text{OCH}_3$
65.8	g5
67.4	
67.8	4, g6
72.1	11
72.9	g3
74.3	g2
79.4	g4
99.1	g1
100.8	
101.0	g7, A
107.6	2', 6'
109.1	8
110.1	5
127.5	4'
130.1	1'
132.7	8''
133.8	4''
146.2	3', 5'
146.4	
148.1	6, 7
174.8	13

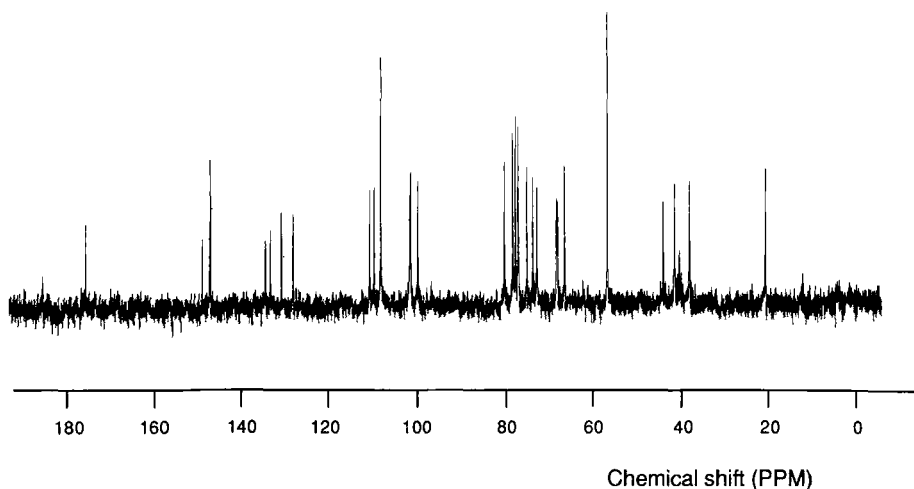


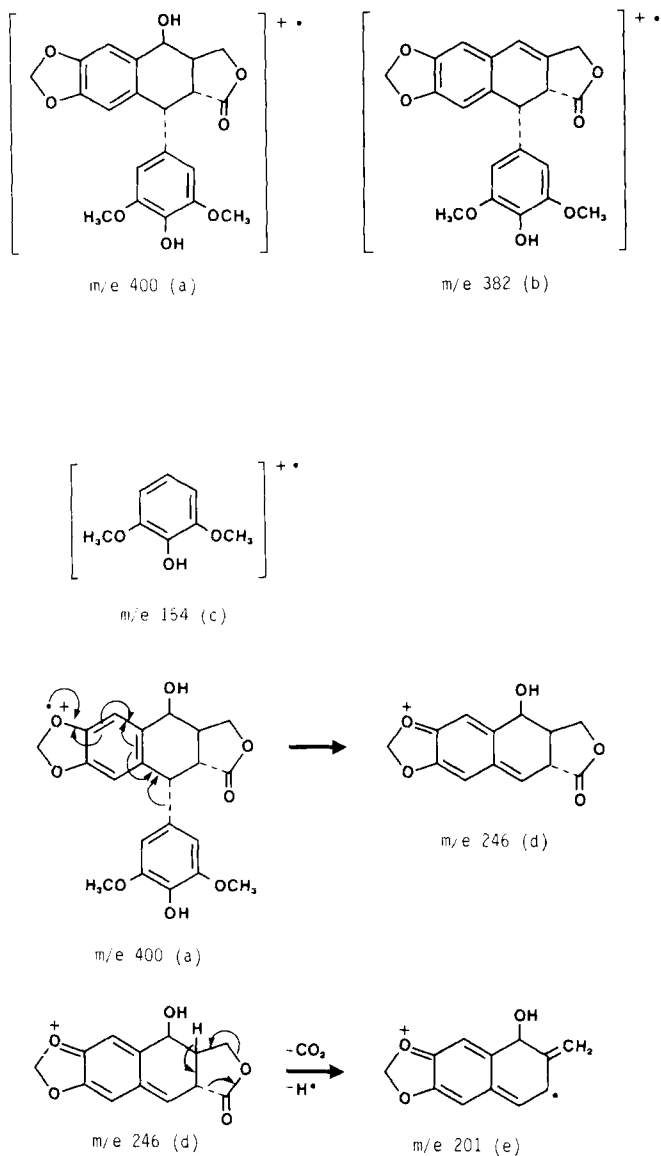
Figure 5.  $^{13}\text{C}$  NMR spectrum of etoposide.

#### 4.5. Mass Spectrum

The electron impact mass spectrum (EI-MS) of etoposide (Figure 6) was measured with a Kratos MS-80 mass spectrometer. The sample was introduced into the ion source (250 °C) by a direct inlet probe. An electron energy of 70 eV and an ionizing current of 100  $\mu\text{A}$  were used. The base peak in the spectrum is the ion at  $m/e$  382. This fragment results from the loss of OH and the glucopyranosyl moiety (structure b, Scheme II).

The fragment corresponding to the loss of only the glucopyranosyl moiety, at  $m/e$  400 (structure a), has a relatively low abundance (3-5%).

An other fragmentation pathway is the formation of a fragment at  $m/e$  154 corresponding to structure c. Minor fragmentation pathways are the formation of fragments at  $m/e$  246 (structure d) and 201 (structure e), both pathways starting from mass 400 (a).



Scheme II. Fragmentation pathways of etoposide.

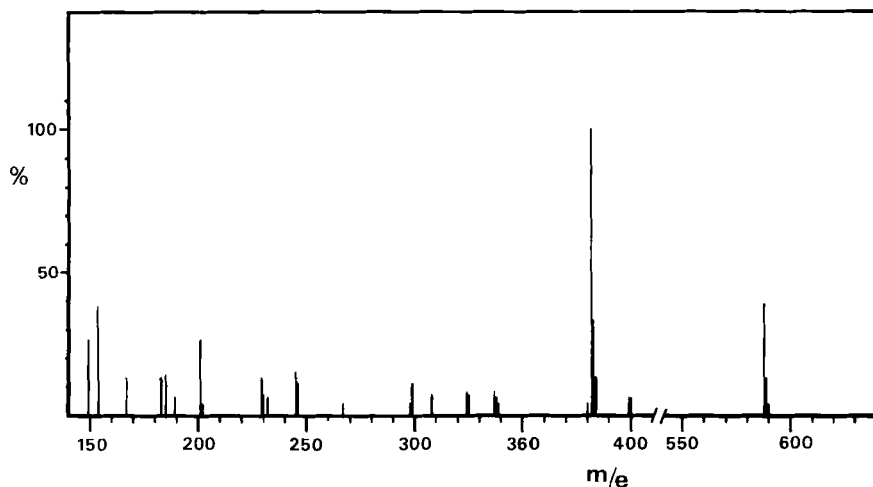


Figure 6. The EI mass spectrum of etoposide.

#### 4.6. Melting Range

The reported melting ranges are:

Etoposide crystallized from methanol: 236-251°C [5].

Etoposide as obtained from the manufacturer: 221-222°C [6].

#### 4.7. Differential Scanning Calorimetry

The DSC thermogram for etoposide (Figure 7) was recorded with a Setaram DSC-III, with a scan rate of 3 K/min. The sample size was about 2 mg. The DSC thermograms were recorded in a nitrogen atmosphere.

An exothermic peak appears between approx. 190 and 210°C (Figure 7), an endothermic peak between 255 and 264°C with a maximum at 258°C (Figure 8).

#### 4.8. Optical Rotation

The optical rotation  $[\alpha]_D^{20}$  of etoposide crystallized from methanol in chloroform ( $C=0.6$  g/v) was  $-110.5^\circ$  [5].



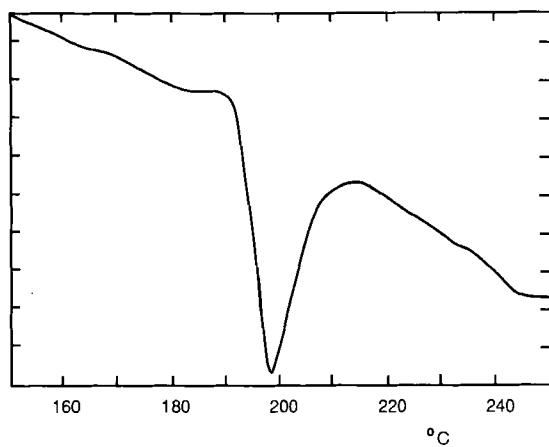


Figure 7. The DSC thermogram of etoposide (exothermic process).

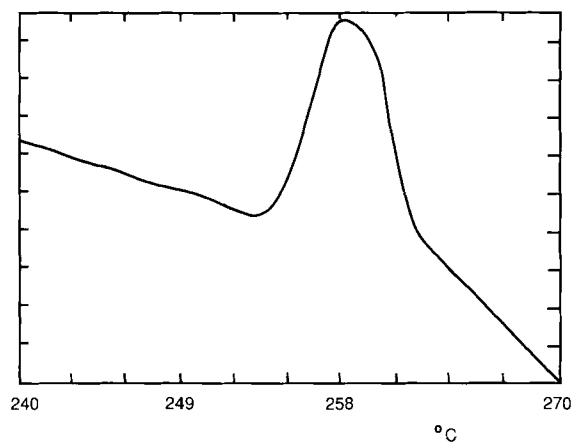


Figure 8. The DSC thermogram of etoposide (endothermic process).

#### 4.9. Dissociation Constant

The pKa of the C4' phenolic function was determined spectrometrically. Spectra of  $10^{-4}$  M solutions of etoposide in 0.05 M sodium borate buffers containing 4% methanol were recorded with a Shimadzu UV-140 double beam spectrometer. The ionic strength was kept at 0.150 M by the addition of KCl. From the inflexion in the plot of the absorbance as a function of pH, a pKa of 9.7 was found [9]. At  $\mu=0.1$  M, the spectrometrically determined pKa was reported to be 9.8 [10].

#### 4.10. Electrochemistry

Etoposide contains a 2,6-dimethoxyphenol group (ring E), which can be oxidized chemically or electrochemically. The oxidation mechanism of etoposide was studied in several aqueous solutions buffered at different pH values [9]. The cyclic voltammogram of etoposide at pH 7.0 is presented in Figure 9. The electrochemical oxidation of etoposide in

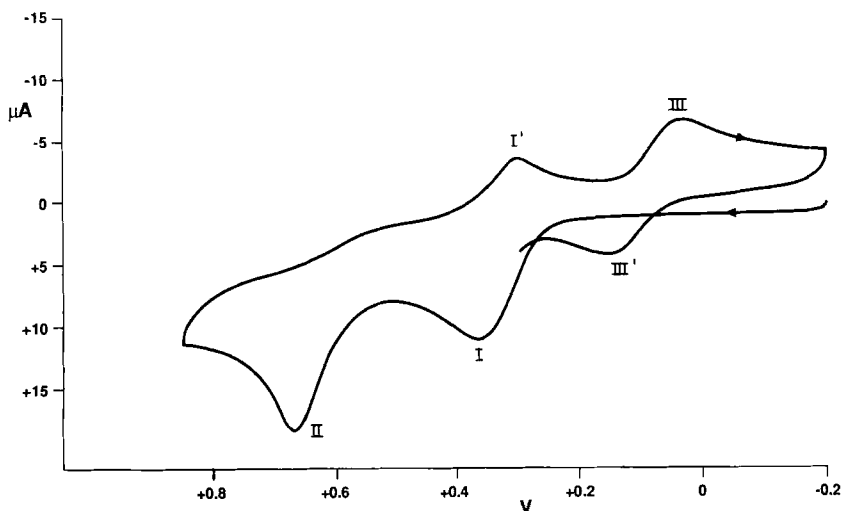


Figure 9. Cyclic voltammogram of 0.3 mM etoposide in 0.1 M phosphate buffer/methanol (90/10 v/v) at a glassy carbon electrode. Scan rate 0.1 V/s. The cyclic voltammogram was recorded from -0.2 V to +0.85 V and to +0.3 V.

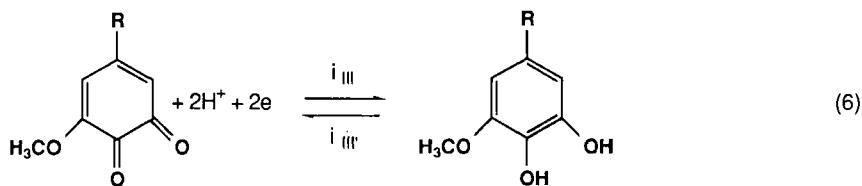
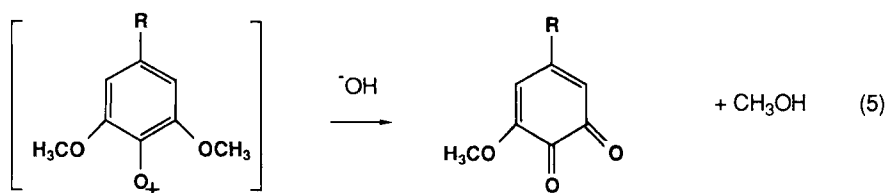
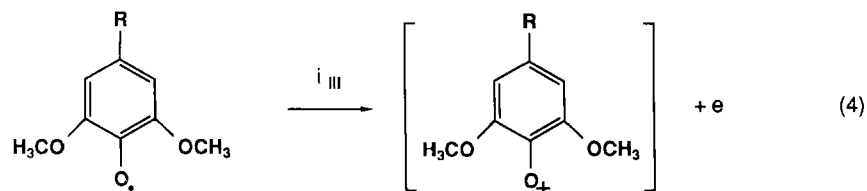
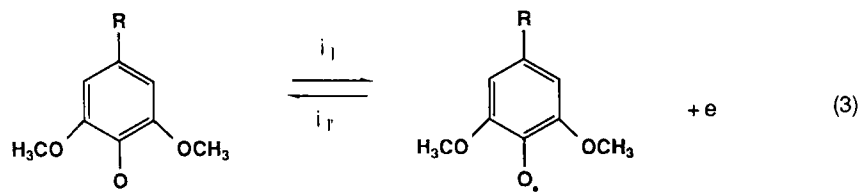
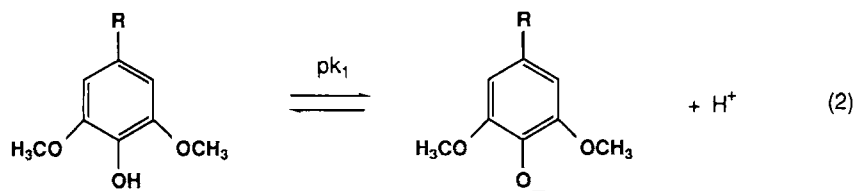
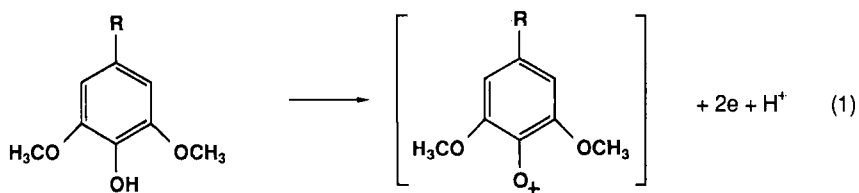


Figure 10. Oxidation mechanism of etoposide.

aqueous solutions shows an overall transfer of two electrons. At pH values below 2.5, the oxidation proceeds in one voltammetric, pH-independent oxidation step (1, Figure 10). At pH values above 2.5, the oxidation proceeds in two voltammetric oxidation steps. The transfer of the first electron (3, Figure 10) is reversible and is preceded by a proton transfer (2, Figure 10). The transfer of the second electron (4) results in the formation of an unstable cation which is converted rapidly into the o-quinone of etoposide (5). The o-quinone is adsorbed at the electrode surface, and is reduced in the cathodic scan ( $i_{111}$ ) to the corresponding hydroquinone. The hydroquinone is oxidized in the second anodic scan ( $i_{111}$ ). Both the oxidation of the hydroquinone and the reduction of the o-quinone are pH-dependent.

## 5. METHODS OF ANALYSIS

### 5.1. Thin Layer and Paper Chromatography

Only a few thin layer chromatographic systems have been described in the literature (see Table IV). Information on paperchromatography is scanty.

Table IV. Thin layer and paper chromatography.

phase	solvent (v/v)	compound	Rf	reference
silicagel	ethyl acetate	etoposide	0.57	11
		<i>cis</i> -etoposide	0.49	
		<i>cis</i> -hydroxy acid		
		of etoposide	0.03	
silicagel	chloroform- methanol (21:1)	etoposide	?	12
	butanol-glacial acetic acid-	etoposide	?	
	water (3:1:1)			
cellulose paper	" "	etoposide	?	12

The spots of etoposide and its degradation products can be detected by irradiation with UV light (254 nm), or the chromatogram can be sprayed with a 50:50 mixture (v/v) of water and 1% ceric ammonium sulfate in 85% phosphoric acid (w/w). The epipodophyllotoxin derivatives appear as orange-red spots [11].

## 5.2. High Performance Liquid Chromatography

The reversed phase HPLC methods published until now were developed for the analysis of etoposide in stability studies (section 6), in bioanalysis (section 8), and in injectable formulations [13]. The HPLC methods used for the analysis of etoposide in stability studies and in injectable formulations are summarized in Table V.

Table V. High performance liquid chromatography of etoposide and its degradation products.

column	mobile phase	detection	reference
RP-8, 5 $\mu$ m (150x4.6 mm i.d.)	acetonitrile- acetic acid -water (27:1:72)	UV, 230, 254 and 286 nm	14
$\mu$ Bondapak Phenyl 10 $\mu$ m (300x4.6 mm i.d.)	0.02 M sodium acetate buffer pH 4-acetonitrile (74:26 v/v)	UV, 200-400 nm	13
$\mu$ Bondapak Phenyl 10 $\mu$ m (300x4.6 mm i.d.)	0.02 M sodium acetate buffer pH 4-acetonitrile (40:60 v/v)	UV, 200-400 nm	13
$\mu$ Bondapak Phenyl 10 $\mu$ m (300x4.6 mm i.d.)	methanol-water (50:50 w/w) con- taining 0.5% (v/w) 0.5 M sodium phos- phate buffer pH 6.5 and 0.5% (w/v) tetrabutylammo- nium bromide solu- tion (20% w/v)	UV, 254 and 280 nm	10

The chromatographic system of Beynen *et al.* [10] separates etoposide, *cis*-etoposide, the *cis*-hydroxy acid of etoposide, and 4'-demethylepipodophyllotoxin. The method of Chow *et al.* [14] separates etoposide from unidentified degradation products. The peak purity of the parent compound is checked by quantification of the parent drug at three wavelengths. Floor *et al.* developed a stability-indicating assay [13] for the determination of the above-mentioned decomposition products, possible synthetic impurities of etoposide, benzyl alcohol, and benzaldehyde in injectable formulations.

## 6. STABILITY AND DEGRADATION OF ETOPOSIDE

### 6.1 Stability in Aqueous Solutions

Etoposide possesses a strained *trans*-lactone ring (Figure 11), which is subject to degradation in acidic and basic media. In acidic media the glucopyranosyl moiety is cleaved yielding 4'-demethylepipodophyllotoxin (aglycon) (I, Figure 11). The aglycon degrades further to the *trans*-hydroxy acid of 4'-demethylepipodophyllotoxin (II).

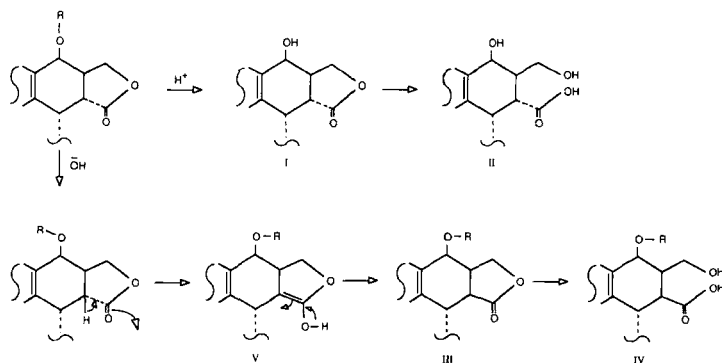


Figure 11. Degradation pathways of etoposide in acidic and alkaline media (R=glucopyranosyl moiety).

At pH values > 5, the degradation of etoposide occurs through epimerization of the *trans*-fused lactone ring to the *cis*-fused lactone (III). Further degradation of this compound results in the formation of the *cis*-hydroxy acid (IV). Conversion of the *trans*-lactone ring into the *cis*-lactone ring (etoposide  $\rightarrow$  III) at pH > 4 occurs through enolization and subsequent conversion of the enol(V) into *cis*-etoposide (III). The last-mentioned reaction requires proton transfers, which are facilitated by bases such as OH<sup>-</sup>, H<sub>2</sub>O, or anions of the acid used as buffer. Etoposide is most stable in the pH region 4-5 [10].

## 6.2. Stability of Etoposide in Plasma

In bioanalysis, chemical instability of etoposide could cause problems. Therefore, the stability of etoposide in plasma was studied at 37, 4 and -18°C. A stability indicating assay was used allowing the quantification of etoposide, *cis*-etoposide and 4'-demethylepipodophyllotoxin, after isolation from biological matrices [6].

Although at 37°C part of the proteins present in plasma precipitate after 6 hours, etoposide itself is stable for at least 72 hours. When stored in plasma at 4 and -18°C, etoposide appeared unchanged for at least 4 and 8 weeks, respectively.

These studies allow the conclusion that it is not necessary to refrigerate plasma samples immediately after preparation [unpublished results].

## 7. PHARMACOLOGY

### 7.1. Mechanism of Action

Etoposide differs in its biological action from its parent podophyllotoxin, which is a spindle poison. Etoposide does not interact with the microtubule assembly [15,16], but prevents cells from entering mitosis. In contrast, the precursor podophyllotoxin arrests cells in the metaphase. Etoposide arrests cells in the late S or G2 phase of the cell cycle and the cells accumulate in the G2 phase. Cells treated with etoposide show a rapid decrease of the mitotic index, with a simultaneous reduction of cell proliferation.

Etoposide has been shown to induce double strand breaks and single strand breaks in DNA in intact cells and in nuclei, but not in purified DNA. The DNA degradation is dose- and temperature-dependent, and reversible after removal of the drug [16-20].

Etoposide is thought to be activated in the cell nucleus by oxidation of the phenolic group to reactive intermediates [21]. Interaction of these intermediates with DNA could also result in DNA damage.

Recent studies indicate that type II topoisomerase is probably the intracellular target in the DNA strand-breaking property of etoposide [22-25].

Etoposide inhibits the cellular uptake of thymidine, uridine, adenosine and guanosine [17,18].

## 7.2. Pharmacokinetics

Upon oral administration to human subjects of hydrophylic capsules or a diluted intravenous preparation, peak etoposide blood levels were seen at 35-240 minutes and 30-160 minutes, respectively [26].

Drug absorption varies within wide limits, the mean values for the biological availability being 57% ( $\pm$  35% s.d.) for the capsules and 91% ( $\pm$  35% s.d.) for the diluted i.v. preparation [26]. Other investigators found an oral absorption after administration of soft gelatine capsules varying from 24.9 to 73.7% (median 48.4%) [27].

The pharmacokinetics of etoposide after i.v. or oral administration is described by an open two-compartment model [26, 27] or an open three-compartment model [28]. No difference is observed in the pharmacokinetics after i.v. administration of high and low doses [26-29].

Figure 12 presents possible metabolic pathways of etoposide, made up from published results from studies on the *in vivo* and *in vitro* metabolism of etoposide.

Pathway A: Low amounts of the *cis* (or *picro*) isomer have been detected by HPLC in plasma, serum [30,31], urine [30, 31], and cerebro spinal fluid [32]. The conversion (pathway A) of etoposide into its *cis* isomer is enhanced by increasing the pH or the temperature [10].

Pathway B: The formation of the *cis*- or *trans*-hydroxy acid of etoposide is a minor metabolic pathway.

Indications for the presence of a metabolite with an opened lactone ring were found *in vitro* [21]. Low amounts were found in patients' urine [28,30,33,34] and plasma [30].



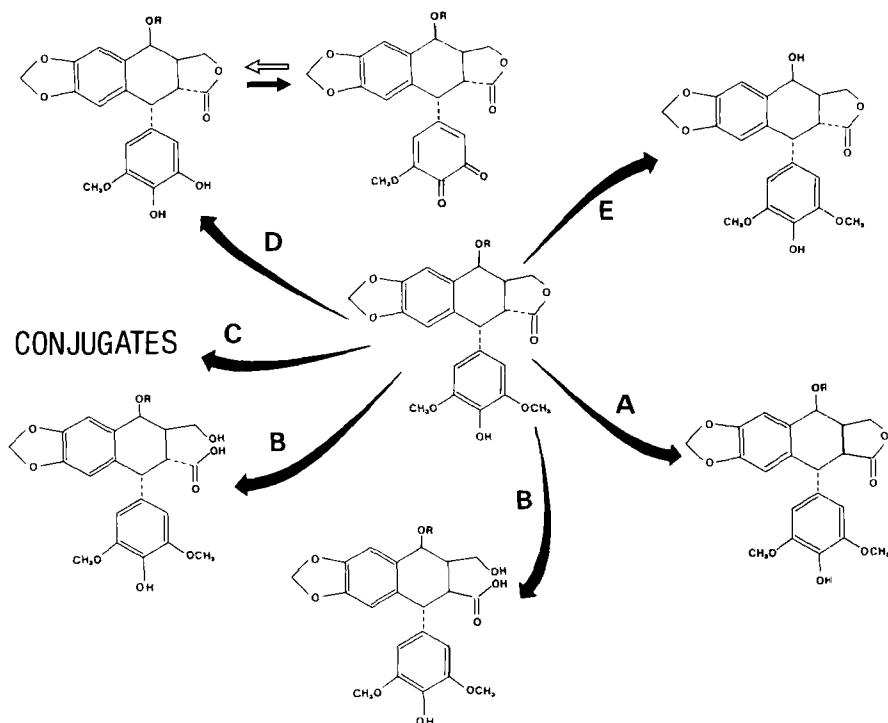


Figure 12. Possible metabolism of etoposide (R = glucopyranosyl moiety).

The major metabolite so far was found to be the glucuronide of etoposide [28,35,36] (Pathway C), the glucuronic acid being attached to the phenol group at C-4' [35]. Glucuronides of etoposide were also isolated from bile of patients [28] and from rat bile [38]. Sulphate conjugates of etoposide (pathway C) could not be detected in patients' urine [28].

After incubation of cellular suspensions with etoposide *in vitro*, two metabolites were found: the reactive *o*-quinone derivative of etoposide and its reduction product, the catechol of etoposide (pathway D) [21,39,41].

In humans, urinary excretion of unchanged drug accounts for an appreciable amount of drug elimination: according to the literature 26.2 - 53.4% of unchanged drug is recovered from urine after intravenous administration [28].

Amounts recovered from the stool (0 - 16.3%) vary as well [40]. After oral administration, 6.4 - 26.2% of the dose was recovered unchanged from the urine within 48 hours [27].

### 7.3. Clinical Activity

In clinical studies, etoposide proved to be active against a variety of tumors. In single agent therapy, etoposide is one of the most active compounds against small cell lung cancer. Other tumors sensitive to etoposide are: testicular cancer, non-Hodgkin lymphoma, neuroblastoma, acute myelomonocytic leukaemia, acute non-lymphocytic leukaemia, and Kaposi's sarcoma [1,41]. In chemotherapy, etoposide is combined with other cytostatics.

### 7.4. Clinical Toxicity

Information on the toxicology of etoposide was reported by O'Dwyer *et al.* [41].

The dose-limiting toxicity of etoposide proved to be dose-related myelosuppression (mainly leucopenia). Bone marrow recovery is usually complete by day 20, and cumulative toxicity has not been reported [42]. Mucositis is an adverse reaction which is observed after high doses of etoposide [28,43].

## 8. ANALYSIS OF ETOPOSIDE AND METABOLITES IN BIOLOGICAL FLUIDS

### 8.1. Analysis of Etoposide

Initially, the disposition of etoposide was studied in humans using a <sup>3</sup>H-labelled (C-4) compound [44]. In the investigations with this labelled drug, the parent compound was separated from non-extractable metabolites and the biological matrices by extraction with chloroform [44,45]. The purity of the extract was checked with several TLC systems, [44,45]. In addition to several chromatographic systems various immunoassays are available for the determination of etoposide [46-49]. Some of these immunoassays are not specific but crossreact with metabolites and degradation products [46,47].

The majority of the analysis methods published for etoposide are carried out with reversed-phase HPLC.

Table VI summarizes these HPLC methods, some of which allow the analysis of metabolites.

Frequently, the parent compound is isolated prior to HPLC analysis by extraction with ethyl acetate [31] or with a halogenated hydrocarbon, either on line [50] or batchwise [6,11,51-60]. Also neutral metabolites and degradation products such as *cis*-etoposide and 4'-demethylepipodophyllotoxin are extracted with these solvents. Only few HPLC systems are able to separate the *cis* isomer from the parent compound [6,31,56, 58-61].

Several internal standards are used, e.g., teniposide [6,11, 51,53,54,55,58,60], 4'-demethylepipodophyllotoxin [50], peltatine (a podophyllotoxin derivative), and the *cis*-hydroxy acid of teniposide [31], diphenylhydantoin or methylphenytoin [52]. Etoposide can also be determined in biological fluids without using an internal standard [56,59,61].

In general, three detection methods are used in the routine determination of etoposide in biological fluids; oxidative electrochemical detection [6,31,56,59-61], fluorescence detection [50,53], and UV detection [11,51,52,54,55,57,58].

The determination limit of the methods is often below 100 ng etoposide per ml plasma [6,31,50,51,53,55-57,59,60] and sometimes above 400 ng per ml plasma [11,52,54,58,61].

Recently a method was published based on flow injection analysis (FIA). Selectivity is obtained by applying UV detection at 365 nm. Prior to UV detection, etoposide is converted electrochemically into the corresponding *o*-quinone [62]. This method allows determination of etoposide, after extraction from plasma with 1,2-dichloroethane, at a level of micrograms per ml plasma.

## 8.2. Etoposide Metabolites

Inspection of the possible metabolic pathways (7.2) shows that etoposide and the various etoposide metabolites differ considerably in their physico-chemical properties such as solubility, extractability, pK value, and stability. The neutral parent compound, the *cis* isomer, 4'-demethylepipodophyllotoxin, the *o*-quinone and other neutral metabolites have similar lipophilic properties. Therefore, these compounds can be extracted from biological fluids with organic solvents such as ethyl acetate and halogenated hydrocarbons, allowing subsequent chromatographic analysis on a reversed-phase HPLC system [6].

Table VI. Published HPLC methods for the analysis of etoposide in biological fluids.

matrix flow	sample pre-treatment	column	detection	determination limit	reference
plasma	Chloroform extraction	Bondapak C18, 10 m	UV 254 nm	500 ng/ml	11
plasma	Chloroform, pre-extraction diisopropylether	Lichrosorb RP 8, 5 m	UV 254 nm	500 ng/ml	54
plasma	Chloroform extraction	Bondapak C18, 10 m	Fluorescence 215/328 nm	50 ng/ml	53
plasma	Chloroform extraction	Partisil ODS	UV 252 nm	100 ng/ml	55
urine	Ethyl acetate extraction	Bondapak Phenyl, 10 m	ECD* + 0.85 V vs Ag/AgCl	20 ng/ml	31
plasma	Preconcentration on PRP.1, post-column extraction with 1,2-dichloroethane	Lichrosorb RP 18, 10 m	Fluorescence 230/328 nm	8 ng/ml 30 ng/ml	50
plasma	Chloroform extraction, wash step with buffer	Lichrosorb RP 18, 10 m	UV 280 nm	30 ng/ml 50 ng/ml	57
plasma	1,2-dichloroethane extraction	Bondapak Phenyl, 10 m	ECD 0.50 V vs Ag/AgCl	2 ng/ml	6,56
serum	Solid-phase extraction C-18 cartridge	Bondapak Phenyl, 10 m	UV 230 nm	400 ng/ml	58
plasma	Chloroform extraction	Bondapak CN, 10 m	ECD +0.50 V vs Ag/AgCl	10 ng/ml	59
plasma	1,2-dichloroethane extraction	Bondapak Phenyl, 10 m	ECD +0.80 V	5 ng/ml	60
plasma	1,2-dichloroethane extraction	Radial-PAK C18, 10 m	Mass spectrometry, UV	unknown	51
plasma	Chloroform extraction	ODS Hypersil 5 m	UV 229 nm	500 ng/ml	52
urine	Solid-phase extraction C-18 Bond Elut	ODS Hypersil 5 m	ECD +0.9 V	500 ng/ml	61

\* ECD = electrochemical detection

The hydroxy acids and the glucuronide(s) are hydrophylic compounds which can not be extracted from aqueous solutions at physiological pH values.

A few methods have been published for the analysis of the hydroxy acids in plasma and urine [11,28,31]. Strife *et al.* [11] used an XAD-4 column for the isolation of the hydroxy acid of etoposide, whereas Sinkule *et al.* [31] isolated the compound from plasma and urine by liquid-liquid extraction with ethyl acetate, after acidification to pH 4. The hydroxy acid can also be determined by direct injection of urine samples [28]. Routine measurement of the glucuronides is carried out by determination of etoposide before and after hydrolysis [28].

#### ACKNOWLEDGEMENT

The authors are gratefull to Mr. D. Seykens, Department of Organic Chemistry, University of Utrecht, The Netherlands for running the NMR spectra and providing relevant <sup>13</sup>C reference data.

## REFERENCES

1. Issel, B.F., Rudolph, A.R., and Louie, A.C. (1984). In: *Etoposide (VP 16). Current Status and New Developments* (B.F. Issel, F.M. Muggia, and S.K. Carter, eds). p. 1. Academic Press Inc., New York.
2. Kelly, M., and Hartwell, J. (1954). *J. Nat. Cancer Inst.* 14, 967.
3. Kuhn, M., and Von Wartburg, A. (1969). *Helv. Chim. Acta* 52, 948.
4. Kuhn, M., Keller-Juslén, C., and Von Wartburg, A. (1969). *Helv. Chim. Acta* 52, 944.
5. Keller-Juslén, C., Kuhn M., and Von Wartburg, A. (1971). *J. Med. Chem.* 14, 936.
6. Holthuis, J.J.M. (1986). Ph.D. Thesis University of Utrecht, The Netherlands.
7. Jardine, I., Strife, R.J., and Kozlowski, J. (1982). *J. Med. Chem.*, 25, 1077
8. Strife, R.J. (1981). Ph.D. Thesis. Purdue University, West Lafayette, Indiana, USA.
9. Holthuis, J.J.M., Zuman, P., Römken, F.M.G.M., Renema, J., and Van Oort W.J. (1985). *J. Electroanal. Chem. Interfacial Electrochem.* 184, 317.
10. Beijnen, J.H., Holthuis, J.J.M., Kerkdijk, H.G., Van der Houwen, O.A.G.J., Paalman, A.C.A., Bult, A., and Underberg, W.J.M., (1988). *Int. J. Pharm.* 41, 169.
11. Strife, R.J., Jardine, I., and Colvin, M. (1980). *J. Chromatogr.* 182, 211.
12. Creaven, P.J. and Allen, L.M. (1975). *Clin Pharmacol. and Ther.* 18, 226.
13. Floor, B.J., Klein, A.E., Muhammad, N., and Ross, D. (1985). *J. Pharm. Sci.* 74, 197.
14. Chow, D., Shak, J., and Chen, J.R. (1987). *J. Chromatogr.* 396, 217.
15. Brewer, C.F., Loike, J.D., and Horwitz, S.B. (1979). *J. Med. Chem.* 22, 215.
16. Loike, J.D. and Horwitz, S.B. (1976). *Biochem.* 15, 5435.
17. Horwitz, S.B. and Loike, J.D. (1977). *Lloydia* 40, 82.
18. Loike, J.D. and Horwitz, S.B. (1976). *Biochem.* 15, 5443.
19. Ross, W., Wozniak, A., Smallwood, S., and Yalowich, J.C. (1984). In: *Etoposide (VP 16). Current Status and New Developments* (B.F. Issel, F.M. Muggia, and S.K. Carter, eds). p. 49. Academic Press Inc., New York.
20. Wozniak, A.J., Glisson, B.S., Hande, K.R., and Ross, W.E. (1984). *Cancer Res.* 44, 626.
21. Van Maanen, J.M.S. (1988) Ph.D. Thesis. Free University, Amsterdam, The Netherlands.

- 22 Chen, G.L., Yang Liu, Rowe, T.C., Halligan, B.D., Tewey, K.M., and Liu, L.F. (1984). *J. Biol. Chem.* 259, 13560.
- 23 Ross, W., Rowe, T., Glisson, B., Yalowich, J., and Liu, L. (1984). *Cancer Res.* 44, 5857.
- 24 Rowe, T., Kupfer, G., and Ross, W. (1985). *Biochem. Pharmacol.* 34, 2483.
- 25 Dorr, R.T., Liddil, J.D., and Gerner, E.W. (1986). *Cancer Res.* 46, 3891.
- 26 D'Incalci, M., Farina, P., Sessa, C., Mangioni, C., Conter, V., Masera, G., Rochetti, M., Brambilla Pisoni, M., Piazza, E., Beer, M., and Cavalli, F. (1982) *Cancer Chemother. Pharmacol.* 7, 141.
- 27 Pfeffer, M., Scalzo, A.J., Nardella, P.A., Comis, R.L., Van Harken, D.R., Issell, B.F., and Smyth, R.D. (1984). In: *Etoposide* (VP 16-213). Current Status and New Developments. (B.F. Issell, F.M. Muggia, and S.K. Carter eds.). p. 127. Academic Press Inc., New York.
- 28 Holthuis, J.J.M., Postmus, P.E., Van Oort, W.J., Hulshoff, A., Verleun, H., Sleyfer, D.Th., and Mulder, N.H. (1986). *Eur. J. Cancer Clin. Oncol.* 22, 1149.
- 29 Hande, K.R., Wedlund, P.J., Noone, R.M., Wilkinson, G.R., Greco, F.A., and Wolff, S.N. (1984). *Cancer Res.* 44, 379.
- 30 Evans, W.E., Sinkule, J.A., Crom, W.R., Dow, L., Look, A.T., and Rivera, G. (1982). *Cancer Chemother. Pharmacol.* 7, 147.
- 31 Sinkule, J.A. and Evans, W.E. (1984). *J. Pharm. Sci.* 73, 164.
- 32 Postmus, P.E., Holthuis, J.J.M., Haaxma-Reiche, H., Mulder, N.H., Vencken, L.M., Van Oort, W.J., Sleijfer, D.Th., and Sluiter, H.J. (1984). *J. Clin. Oncol.* 2, 215.
- 33 Allen, L.M., Marcks, C., and Creaven, J. (1983). *Proc. Amer. Assoc. Cancer Res.* 25, 131.
- 34 Sinkule, J.A., Hutson, P., Hayes, F.A., Etcubanas, E., and Evans, W. (1984). *Cancer Res.* 44, 3109.
- 35 Holthuis, J.J.M., Van Oort, W.J., and Hulshoff, A. (1986). Bioactive Analytes, Including CNS Drugs, Peptides and Enantiomers. In: *Methodological Surveys in Biochemistry and Analysis*. (Ed. E. Reid).p. 389. Plenum Publishing Corporation, New York, London.
- 36 D'Incalci, M., Rossi, C., Zucchetti, M., Urso, R., Cavalli, F., Mangioni, C., Willems, Y., and Sessa, C. (1986). *Cancer Res.* 49, 2566.
- 37 D'Incalci, M., Sessa, C., Farina, P., Rossi, C., Beer, M., Cavalli, F., Masera, G., and Mangioni, C. (1982). *Proc. Am. Assoc. Cancer Res.* 23, 131.

- 38 Colombo, T., D'Incalci, M., Farina, P., Rossi, C., Guaitani, A., Bartosek, I., Benfenati, E., Fanelli, R., and Garrattini, S. (1983). *Proc. Amer. Assoc. Cancer Res.* 24, 292.
- 39 Sinha, B.K., Trush, M.A., and Kalyanaraman, B. (1984). *Proc. Amer. Assoc. Cancer Res.* 25, 354.
- 40 Creaven, P.J. (1982). *Cancer Chemother. Pharmacol.* 7, 133.
- 41 O'Dwyer, P.J., Leyland-Jones, B., Alonso, M.T., Marsoni, S., and Wittes, R.E. (1985). *New Engl. J. Med.* 312, 692.
- 42 Philips, N.C. and Lauper, R.D. (1983). *Clin. Pharm.* 2, 112.
- 43 Wolff, S.M., Fer, M.F., Mackay, C., Hainsworth, J., Hande, K.R., and Greco, F.A. (1982). *Proc. Amer. Assoc. Cancer Res.* 23, 134.
- 44 Allen, L.M. and Creaven, P.J. (1975). *Europ. J. Canc. Clin. Oncol.* 11, 679.
- 45 Pelsor, F.R., Allen, L.M., Creaven, P.J. (1978). *J. Pharm. Sci.* 67, 1106.
- 46 Ho, D.H., Kannellopoulos, K.S., Brown, N.S., Issell, B.F., and Bodey, G.F. (1985). *J. Immunol. Methods.* 85, 5.
- 47 Aherne, G.H. and Marks, V. (1982). *Cancer Chemother. Pharmacol.* 7, 117.
- 48 Hacker, M.P., Keve, S., and Ershler, W.B. (1984). *Proc. Amer. Assoc. Cancer Res.* 25, 364.
- 49 Yamashita, K., Watanabe, K., Taka-Yama, H., Ishibashi, M., and Miyazaki, H. (1987). *J. Pharm. Biomed. Anal.* 5, 11.
- 50 Werkhoven-Goewie, C.E., Brinkman, U.A.Th., Frei, R.W., De Ruiter, C., and De Vries, J. (1983). *J. Chromatogr.* 276, 349.
- 51 Danigel, H., Pflüger, K-H., Jungclas, H., Schmidt, L., and Dellbrügge, J. (1985). *Canc. Chemother. Pharmacol.* 15, 121.
- 52 Harvey, V.J., Joel, S.P., Johnston, A., and Slevin, M.L. (1985). *J. Chromatogr.* 339, 419.
- 53 Strife, R.J., Jardine, I., and Colvin, M. (1981). *J. Chromatogr.* 224, 168.
- 54 Farina, P., Marzillo, G., and D'Incalci, M. (1981). *J. Chromatogr.* 222, 141.
- 55 Allen, L.M. (1980). *J. Pharm. Sci.* 69, 1440.
- 56 Holthuis, J.J.M., Römken, F.M.G.M., Pinedo, H.M., and Van Oort, W.J. (1983). *J. Pharm. Biomed. Anal.* 1, 89.
- 57 Holthuis, J.J.M., Van Oort, W.J., and Pinedo, H.M. (1981). *Anal. Chim. Acta* 130, 23.
- 58 Hersh, M.R. and Ludden, T.M. (1986). *J. Pharm. Sci.* 75, 815.



- 59 Duncan, G.F., Farmen, R.H., Movahhed, H.S., and Pittmann, K.A. (1986). J. Chromatogr. 380, 357.
- 60 Littlewood, T.J., Hutchings, A.L., Bentley, D.P., and Spragg, B.P. (1984). J. Chromatogr. 336, 434.
- 61 Rideout, J.M., Ayres, D.C., Lim, C.K., and Peters, T.J. (1984). J. Pharm. Biomed. Anal. 2, 125.
- 62 Van Opstal, M.A.J., Blauw, J.S., Holthuis, J.J.M., Van Bennekom, W.P., and Bult, A. (1987). Anal. Chim. Acta 202, 35.

*Analytical Profile of Furosemide*

*By*

*Abdulrahman Mohammad Al-Obaid, Fahad Jaber Al-Shammary\*,  
Khalid Abdul Mohsen Al-Rashood and Mohammad Saleem Mian*

*Department of Pharmaceutical Chemistry, College of Pharmacy,  
King Saud University, Riyadh, Saudi Arabia*

*\*Department of Clinical Laboratories, College of Applied  
Medical Sciences, King Saud University, Riyadh,  
Saudi Arabia*

## Contents

1. Description
  - 1.1 Nomenclature
    - 1.1.1 Chemical Names
    - 1.1.2 Generic Names
  - 1.2 Formulae
    - 1.2.1 Empirical
    - 1.2.2 Structural
    - 1.2.3 CAS Registry Number
  - 1.3 Molecular Weight
  - 1.4 Elemental Composition
  - 1.5 Appearance, Color, Odor and Taste
2. Physical Properties
  - 2.1 Melting Range
  - 2.2 Solubility
  - 2.3 pH
  - 2.4 Stability
  - 2.5 Crystal Structure
  - 2.6 Spectral Properties
    - 2.6.1 Ultraviolet Spectrum
    - 2.6.2 Infrared Spectrum
    - 2.6.3 Nuclear Magnetic Resonance Spectra
      - 2.6.3.1 Proton Spectrum
      - 2.6.3.2  $^{13}\text{C}$  NMR Spectra
    - 2.6.4 Mass Spectrum
3. Synthesis
  - 3.1 Route 1
  - 3.2 Route 2
  - 3.3 Route 3
  - 3.4 Route 4
  - 3.5 Route 5
  - 3.6 Route 6
  - 3.7 Route 7
  - 3.8 Route 8
  - 3.9 Route 9
  - 3.10 Route 10
  - 3.11 Route 11
4. Absorption and Elimination

## 5. Methods of Analysis

### 5.1 Elemental Analysis

### 5.2 Identification Tests

### 5.3 Titrimetric Methods

### 5.4 Spectrophotometric Methods

#### 5.4.1 Ultraviolet

#### 5.4.2 Colorimetric Methods

#### 5.4.3 Nuclear Magnetic Resonance

### 5.7 Chromatographic Methods

#### 5.5.1 Column Chromatography

#### 5.5.2 Gas Liquid Chromatography

#### 5.5.3 Thin-Layer Chromatography

#### 5.5.4 High Performance Liquid Chromatography

## 6. Acknowledgements

## 7. References

## Furosemide

### 1. Description

#### 1.1 Nomenclature

##### 1.1.1 Chemical Names

a) 5-(Aminosulfonyl)-4-chloro-2-[(2-furanyl-methyl)amino]benzoic acid. (1)

b) 4-Chloro-N-furfuryl-5-sulfamoyl-anthranilic acid. (2).

c) 4-Chloro-N-(2-Furylmethyl-5-sulfamoylanthranilic acid. (3).

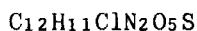
d) 4-Chloro-2-furfurylamino-5-sulphamoyl benzoic acid. (4).

##### 1.1.2 Generic Names

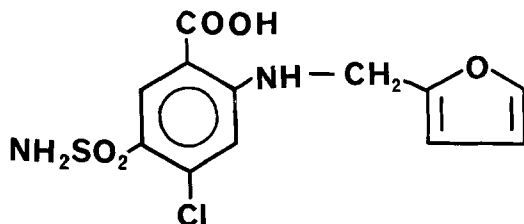
Frusemide, Fursemide, Aisemide, Beronald, Desdemin, Diural, Dryptal, Errolon, Frusemin, Fulsix, Fuluromide, Furosemide, Mita, Furosedon, Katlex, Lasilix, Lasix, Lowpstron, Macasirool, Nicorol, Profemin, Rosemide, Transit, Trofurit, Urosemide, Urex.

#### 1.2 Formulae

##### 1.2.1 Empirical



##### 1.2.2 Structural



1.2.3 CAS Registry  
[54-31-9]

1.3 Molecular Weight

$$\text{C}_{12}\text{H}_{11}\text{ClN}_2\text{O}_5\text{S} = 330.77$$

1.4 Elemental Composition

C, 43.57% H, 3.35%, Cl 10.72% N, 8.47%, O, 24.19%, S, 9.70%.

1.5 Appearance, Color, Odor and Taste

A white to slightly yellow, odorless, almost tasteless crystalline powder.

2. Physical Properties

2.1 Melting Range

M.p. : 206°C

2.2 Solubility

It is slightly soluble in water and chloroform and ether (3). Soluble in acetone, methanol, dimethyl formamide (1) and in solutions of alkali hydroxides (3).

2.3 pH (4)

pH of aq. solutions is in between 8.9 to 9.3.

2.4 Stability

Furosemide injection should be stored at temperature of 15-30°C and protected from light, injections having yellow color should not be used. Exposure of furosemide tablets to light may cause discoloration, discolored tablets should not be dispensed. Tablets should be stored and dispensed in well closed, light resistant containers. Commercially available furosemide tablets have an expiration date of 5 years and commercially available injections has an expiration date of 42 months following the date of

manufacture. Furosemide oral solution should be stored at 15-30°C and protected from light and freezing; once opened unused portion should be discarded after 60 days.

Furosemide injections can usually be mixed with weakly alkaline and neutral solutions having pH of 7-10, such as 0.9% sodium chloride injection or Ringer's injection and some weakly acidic solutions having a low buffer capacity. The injection should not be mixed with strongly acidic solutions (i.e. pH less than 5.5) such as those containing ascorbic acid, tetracycline, epinephrine, norepinephrine, because furosemide may be precipitated. Other drugs which should not be mixed with furosemide injections include most salts of organic bases including local anesthetics, alkaloids, antihistamines, hypnotics, meperidine, and morphine (5).

## 2.5 Crystal Structure (6)

The crystal structure of furosemide, is triclinic,  $P\bar{1}$ , with cell dimensions  $a = 5.234(3)$ ,  $b = 8.751(6)$ ,  $c = 15.948(15)$  Å,  $\alpha = 103.68(12)$ ,  $\beta = 69.94(9)$ ,  $\gamma = 95.99(12)(12)^\circ$  and  $Z = 2$ . The structure was solved by direct methods and refined to  $R = 0.11$  for the 1221 observed reflections measured with  $\text{CuK}\alpha$  radiation on a diffractometer. The furan ring is disordered in this structure, which indicates that the furanyl moiety of furosemide is conformationally labile. The molecules related by a center of symmetry and translations along the  $a$  direction are linked by the  $\text{NH} \cdots \text{O}$  hydrogen bonds to form a column. Between these columns there are only van der Waals interactions.

A projection of the crystal packing of furosemide along the  $a$  axis is presented in Fig. (1). There are three unique hydrogen bonds (one intra- and two intermolecular) in this structure which are tabulated in table (1). The selected bond lengths (Å) and angles ( $^\circ$ ) are listed in table (2).

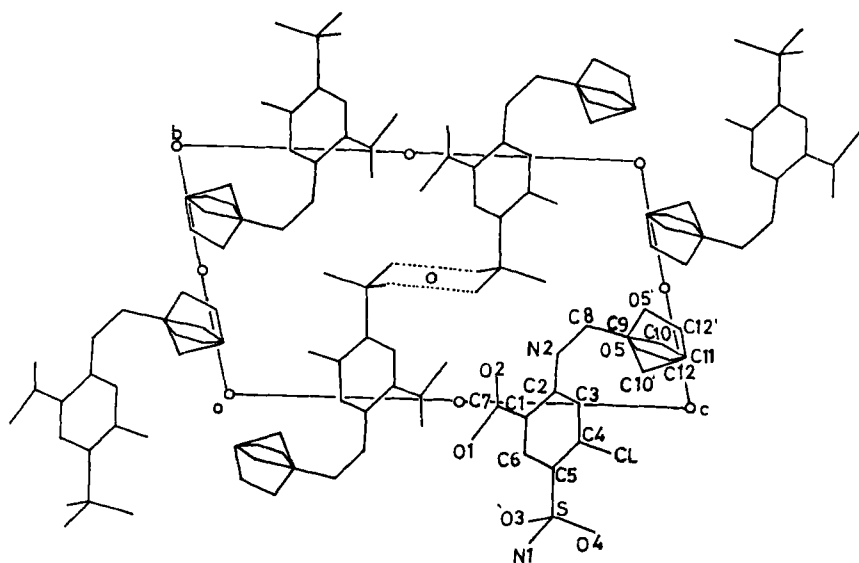


Fig. 1: The molecular packing diagram of furosemide.



Table 1. Hydrogen Bonds in Furoseamide

a	b	c	a-c(Å)	b-c(Å)	∠abc	Position of c
Intramolecular						
N(2)-H.....O(2)			2.71	2.3	107	x,y,z
Intramolecular						
N(1)-H(1)...O(3)			3.00	1.9	163	-x, -l-y, l-z
N(1)-H(2)...O(3)			2.97	2.3	114	l + x,y,z

Table 2. Selected Bond Lengths (Å) and Angles (°) in Furoseamide

C(1)-C(7)	1.50(2)	C(2)-N(2)	1.40(1)	C(4)-Cl	1.73(1)
C(5)-S	1.78(1)	S-O(3)	1.45(1)	S-O(4)	1.40(1)
S-N(1)	1.62(1)	C(7)-O(1)	1.29(2)	C(7)-O(2)	1.24(2)
N(2)-C(8)	1.47(2)	C(8)-C(9)	1.44(2)	C(9)-O(5)	1.46(3)
C(9)-C(10)	1.44(4)	C(10)-C(11)	1.25(4)	C(11)-C(12)	1.26(4)
C(12)-O(5)	1.35(4)	C(9)-O(5')	1.39(3)	C(9)-C(10')	1.38(5)
C(10')-C(11)	1.61(5)	C(11)-C(12')	1.31(4)	C(12')-O(5')	1.40(5)
N(1)-S-C(5)	106.9(6)	O(3)-S-O(4)	118.9(6)		
O(3)-S-N(1)	105.8(7)	O(3)-S-C(5)	108.0(6)		
O(4)-S-N(1)	108.6(7)	O(4)-S-C(5)	107.9(6)		
S-C(5)-C(6)	117.8(3)	S-C(5)-C(4)	122.1(3)		
Cl-C(4)-C(5)	121.8(3)	Cl-C(4)-C(3)	118.1(3)		
C(7)-C(1)-C(6)	116.3(9)	C(7)-C(1)-C(2)	123.7(9)		
C(1)-C(2)-N(2)	121.6(6)	C(3)-C(2)-N(2)	118.3(6)		
O(2)-C(7)-C(1)	121(2)	O(1)-C(7)-C(1)	114(2)		
O(1)-C(7)-O(2)	125(1)	N(2)-C(8)-C(9)	114(1)		
C(8)-C(9)-O(5)	124(2)	C(8)-C(9)-C(10)	129(2)		
O(5)-C(9)-C(10)	94(2)	C(9)-C(10)-C(11)	114(2)		
C(10)-C(11)-C(12)	113(3)	C(11)-C(12)-O(5)	106(3)		

## 2.6 Spectral Properties

### 2.6.1 Ultraviolet Spectrum (7)

The UV spectrum of furosemide in H<sub>2</sub>O was scanned from 190 to 400 nm using DMS 90 Varian spectrophotometer (Fig. 2). It exhibited a  $\lambda_{\max}$  at 228 nm. Other reported UV spectral data are shown in Table 3.

Table 3

<u>Solvent</u>	<u><math>\lambda_{\max}</math> nm</u>	<u>E1%, 1 cm</u>
Ethanol (95%)	288	945
	276	588
	336	144
0.1 N NaOH	226	1147
	273	557
	336	133

---

### 2.6.2 Infrared Spectrum (7a)

The infrared absorption spectrum of furosemide as KBr-disc was recorded on a Perkin Elmer 580 B Infrared Spectrophotometer (Fig. 3). The spectral assignments are listed in Table (4).

Table 4

<u>Frequency cm<sup>-1</sup></u>	<u>Type of vibration</u>	<u>Assignment</u>
3350-3400	NH	C-NH
1671	C = O	- COOH group
1596	NH	- NH <sub>2</sub> group
1322	- S = O	- SO <sub>2</sub> group
582	Cl	C - Cl

---

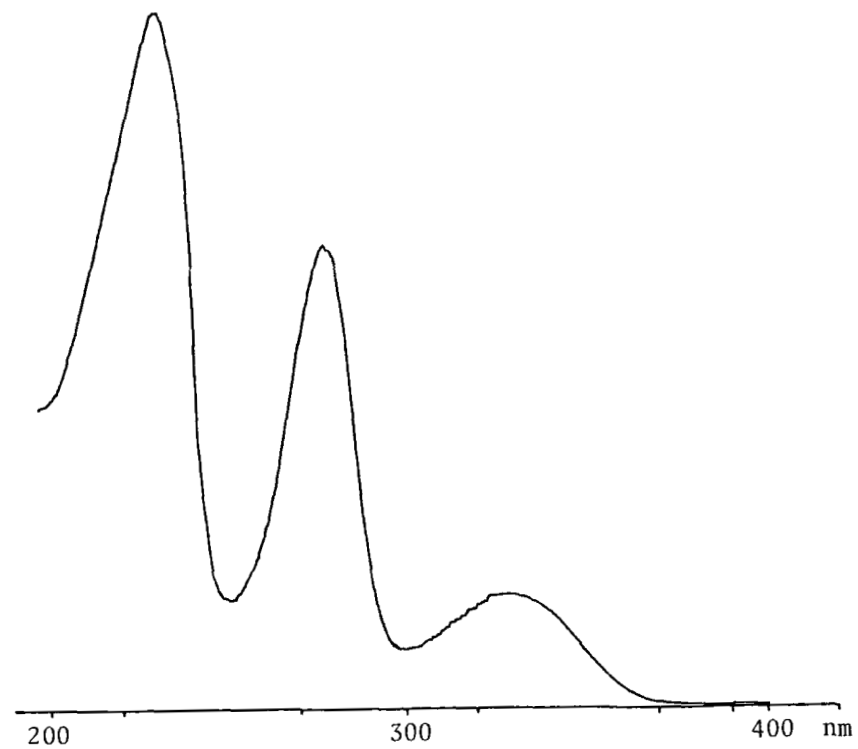


Fig. 2: UV spectrum of furosemide in  $\text{H}_2\text{O}$ .

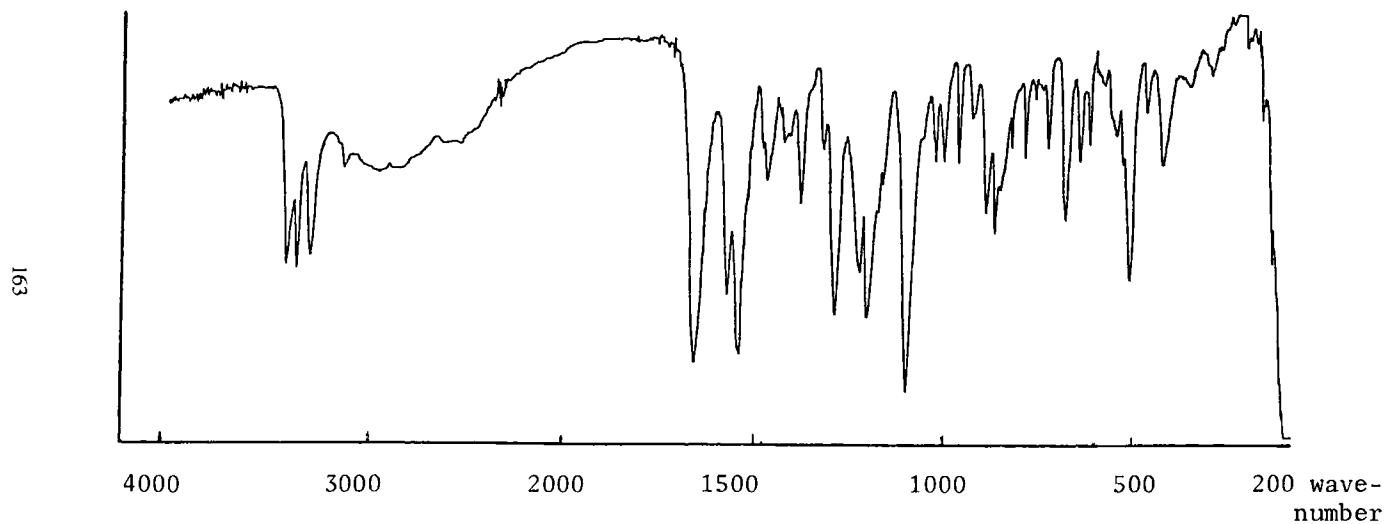


Fig. 3: I.R. spectrum of furosemide as KBr disc.

### 2.6.3 Nuclear Magnetic Resonance Spectra

#### 2.6.3.1 Proton Spectrum (7b)

The PMR spectrum of furosemide in DMSO-d<sub>6</sub> (Fig. 4) was recorded on a Varian (FT 80 A). NMR spectrophotometer using TMS as internal standard. The PMR spectrum of furosemide is unique in a way that all peaks appear as sharp singlets. Chemical shifts are shown in Table 5.

Table (5). PMR characteristic of Furosemide.

<u>Group</u>	<u>Chemical Shift (δ) ppm</u>
-CH <sub>2</sub>	4.5
H <sub>3</sub> and H <sub>4</sub> of the furan ring.	6.41
H <sub>2</sub> of the furan ring	7.24
H <sub>3</sub> of phenyl group	7.32
H <sub>6</sub> of phenyl group	8.42

#### 2.6.3.2 <sup>13</sup>C NMR Spectra (7c)

The <sup>13</sup>C NMR spectrum of furosemide in DMSO-d<sub>6</sub> using TMS as an internal reference is recorded on a Varian XL 200 MHz NMR spectrometer and is presented in Fig. 5.

#### 2.6.4 Mass Spectrum (7d)

The mass spectrum of frusemide obtained by electron impact ionization (EI) is shown in (Fig. 6). It was recorded on a Varian MAT 311 mass spectrometer. The spectrum was scanned from 30 to 400 a.m.u. Electron energy was 70 ev. Emission current 300 μA and ion source pressure 10<sup>-6</sup> Torr. The mass spectral data are shown in Table (6).

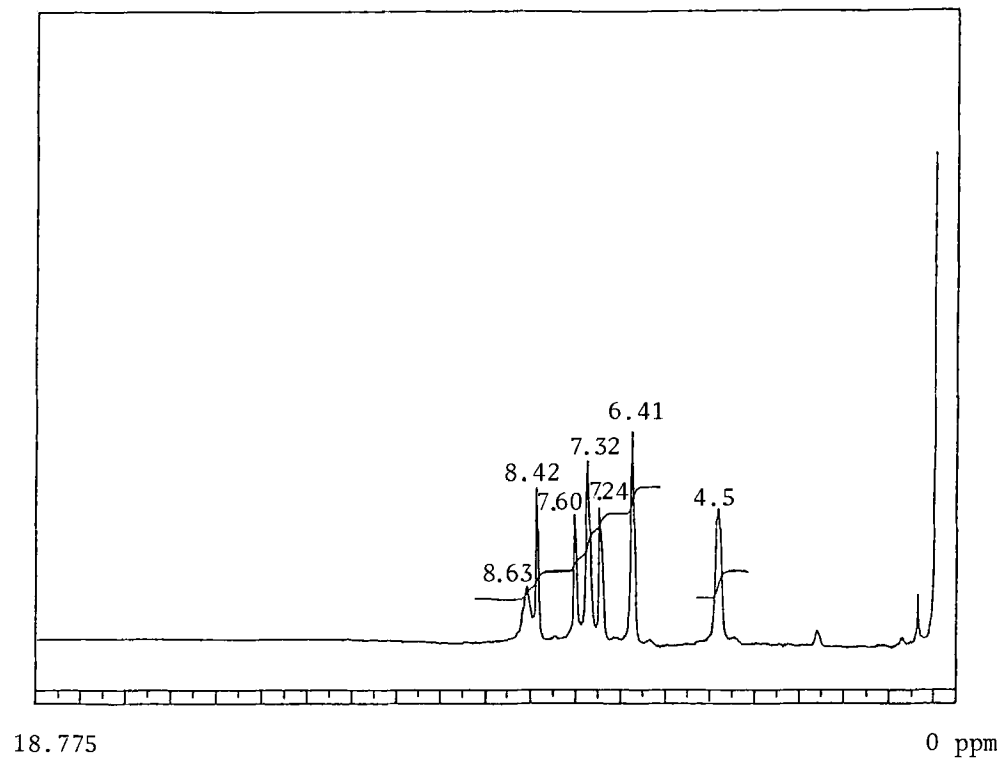


Fig. 4: PMR spectrum of furosemide in DMSO-d<sub>6</sub>.

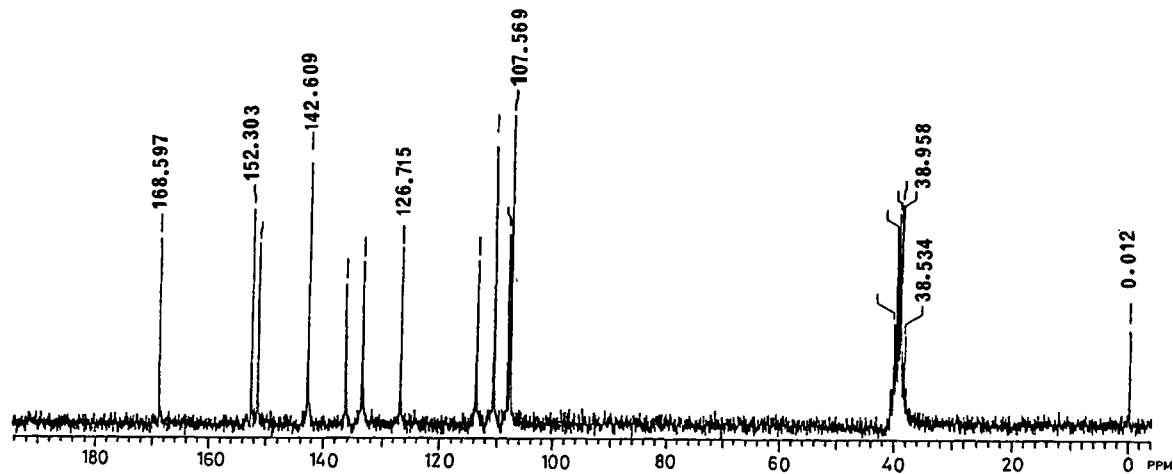


Fig. 5:  $^{13}\text{C}$ -NMR spectrum of furosemide in  $\text{DMSO-d}_6$ .

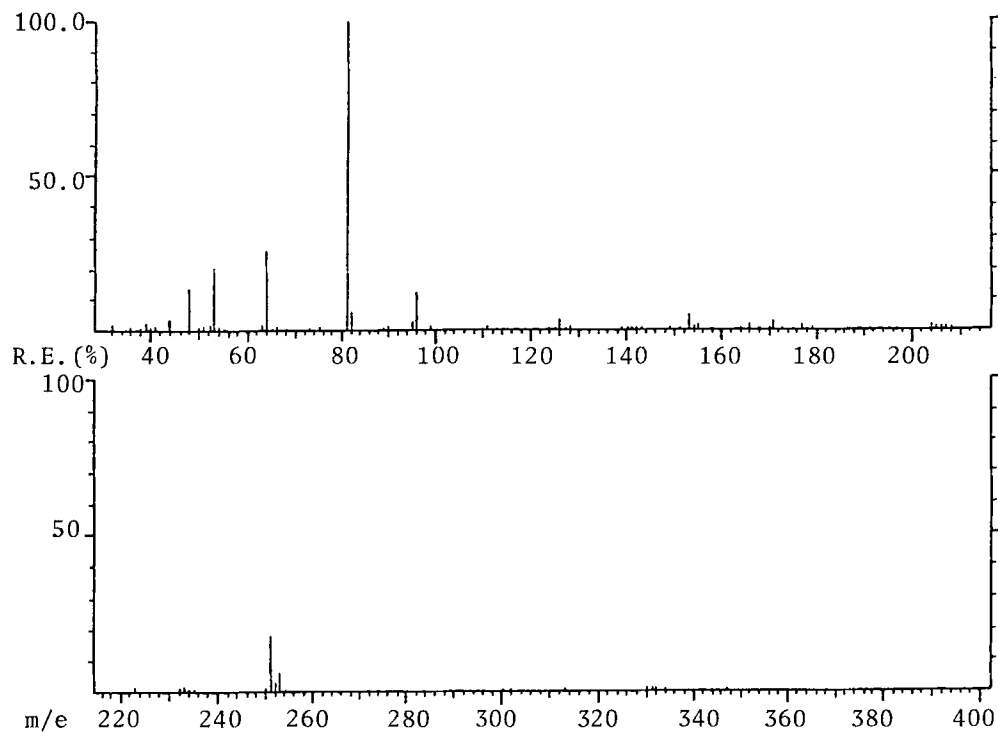
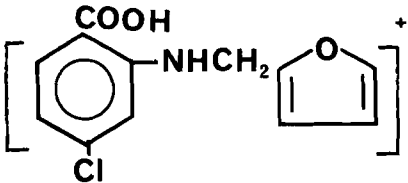
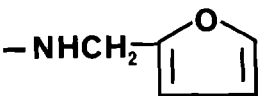
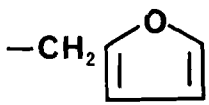


Fig. 6: EI-mass spectrum of furosemide.



Table (6). The most prominent fragments of furoseimide

<u>m/e</u>	<u>Relative intensity</u> %	<u>Fragment</u>
251	20	
96	14	
81	100	
64	38	SO <sub>2</sub>
53	22	—
48	15	— SO

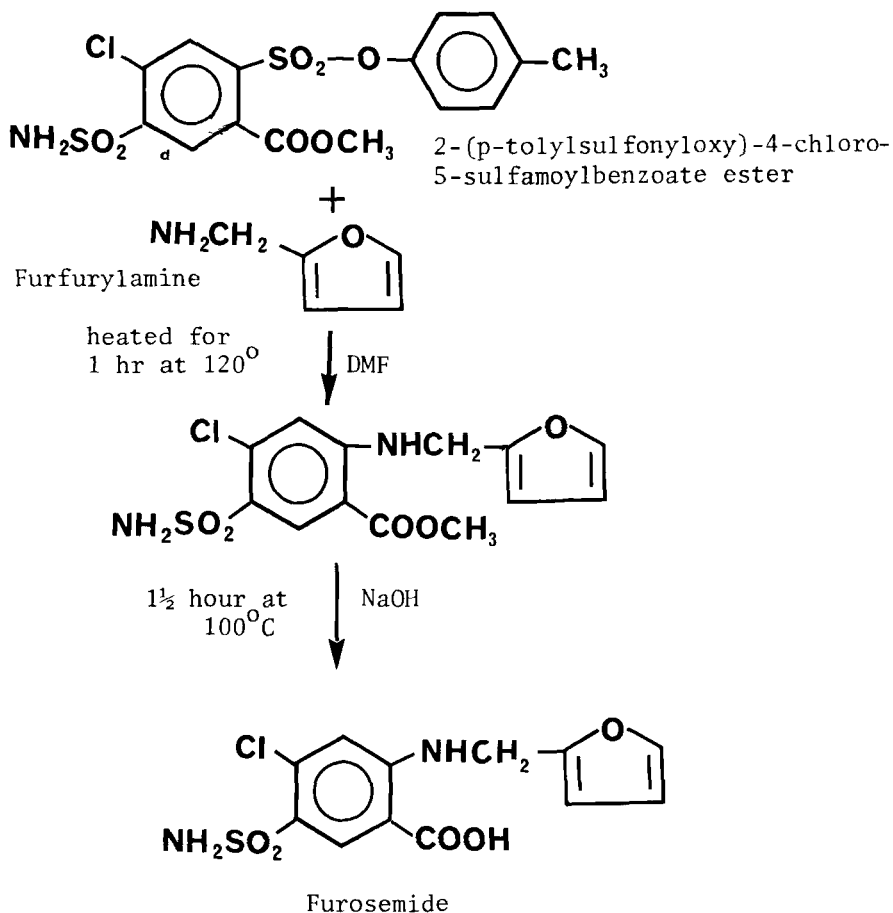
---

3. Synthesis

Furosemide can be synthesized by different routes:

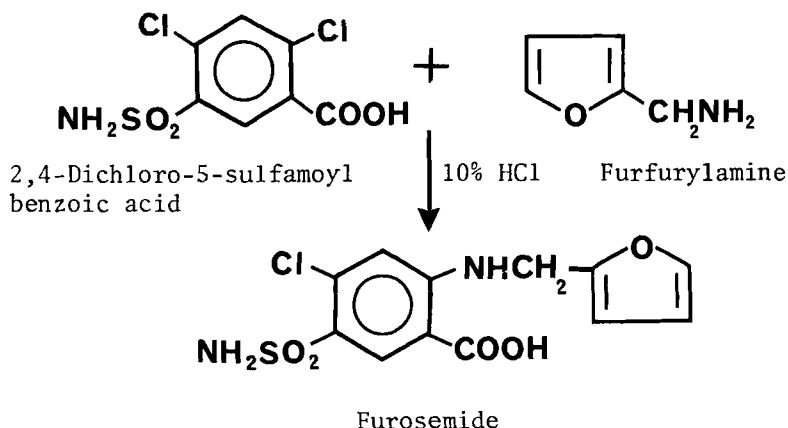
Route 1

The furosemide 2-furfurylamino-4-chloro-sulfamoylbenzoic acid was prepared by treating 2-(p-tolylsulfonyloxy)-4-chloro-5-sulfamoylbenzoate ester with furfurylamine and subsequent hydrolysis of the ester (8).

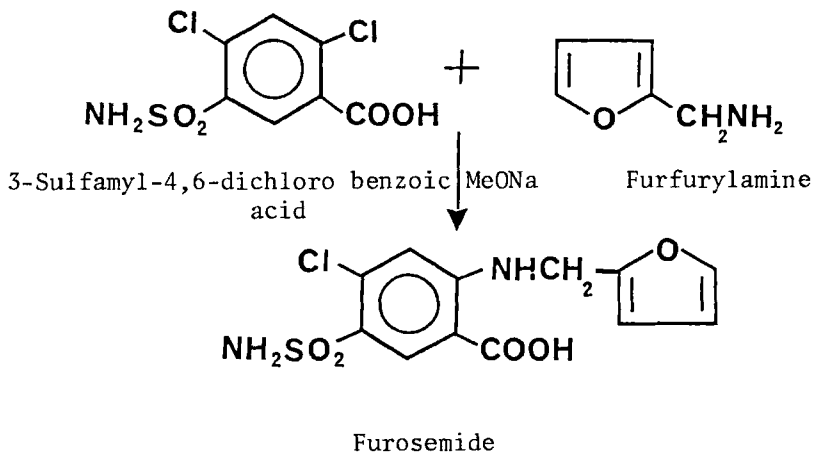


Route 2

2,4-Dichloro-5-sulfamoylbenzoic acid is treated with furfurylamine. To decrease the furfurylamine oxidation, the reaction is done in N<sub>2</sub> atmosphere. Product is obtained from alkaline solution by addition of 10% solution of HCl. The residual furfurylamine is separated by alkali addition (9).

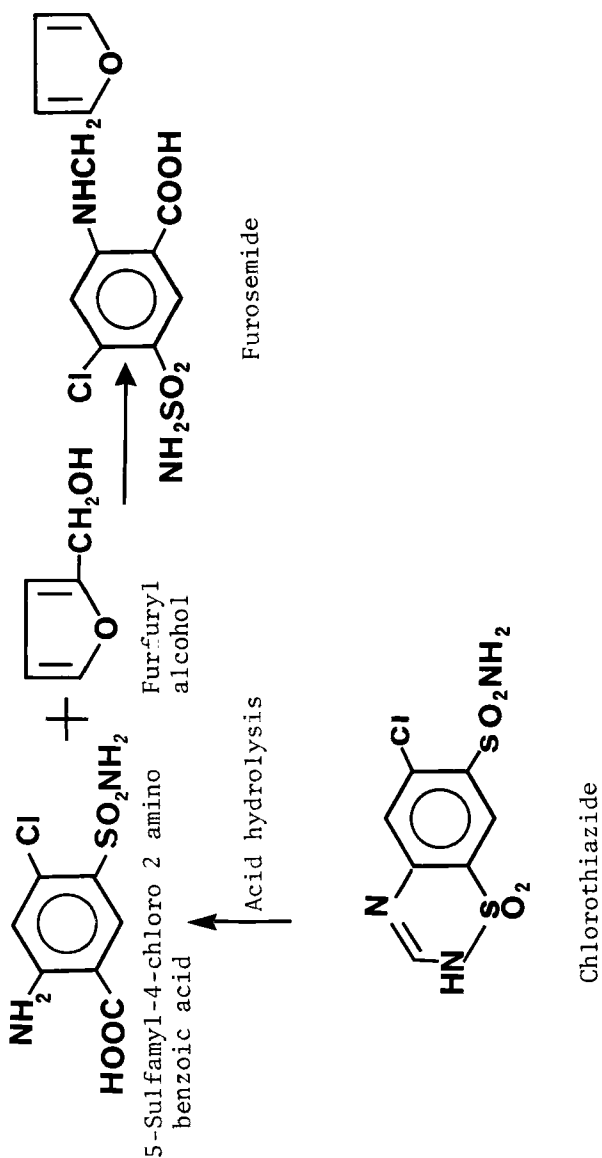
Route 3

3-Sulfamoyl-4,6-dichlorobenzoic acid was treated with MeONa followed by furfurylamine to give 4-chlorofurfuryl-5-sulfamoylanthranilic acid (10).



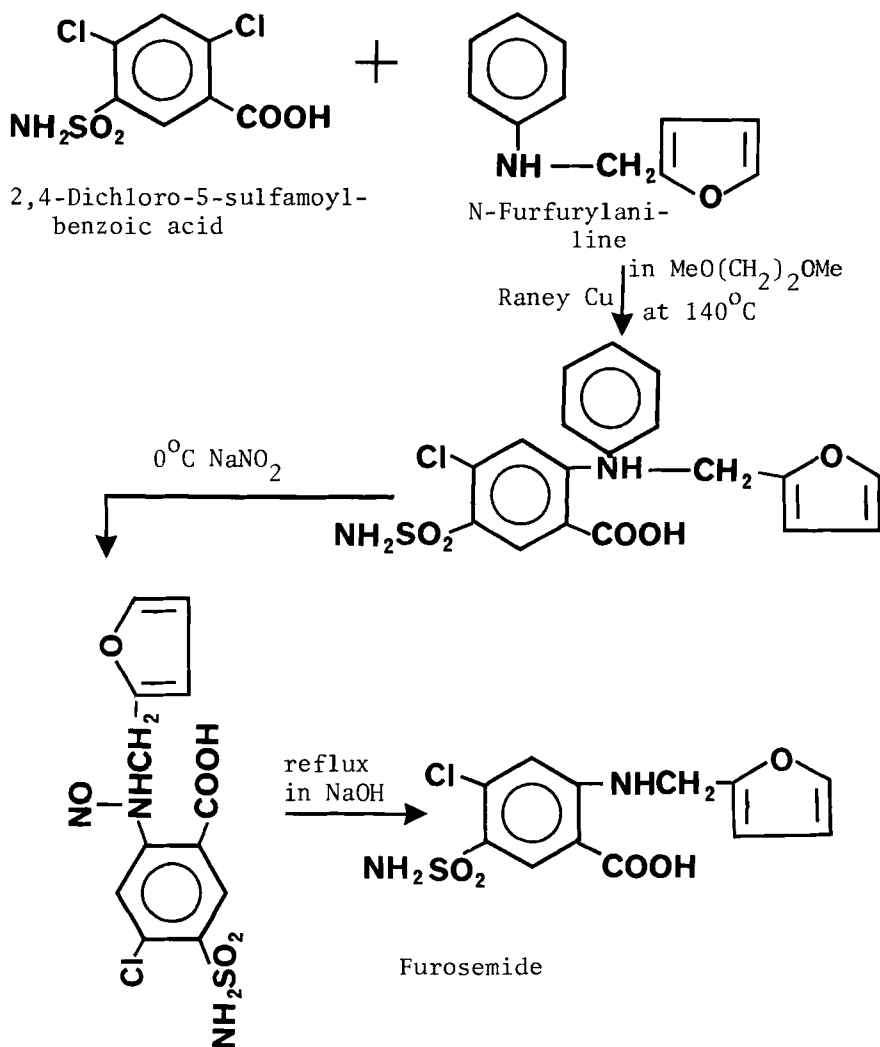
Route 4 (11)

Acid hydrolysis of chlorothiazide and furfuryl alcohol.



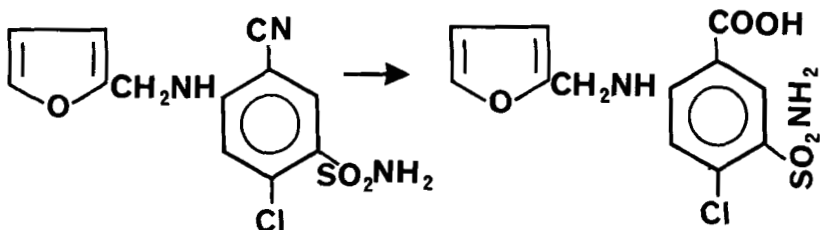
## Route 5 (12)

2,4-Dichloro-5-sulfamoylbenzoic acid was treated with N-furfurylaniline and Raney Cu in  $\text{MeO}(\text{CH}_2)_2\text{OMe}$  (dimethoxy ethane) at  $140^\circ$ . The product dissolved in dichloroethane, extracted with N NaOH, and precipitated with  $\text{AcOH-HCl}$ . The precipitate in 10% aq. HCl treated with  $\text{NaNO}_2$  at  $0^\circ$  and refluxed in NaOH to give furoseimide.

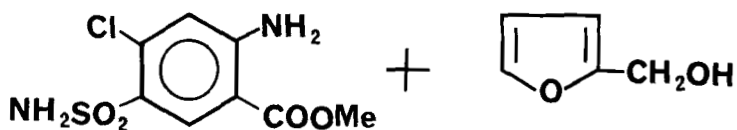


Route 6 (13)

Furosemide is prepared by saponification of corresponding nitriles with aq. NaOH at 75° for 4 hours.

Route 7 (14)

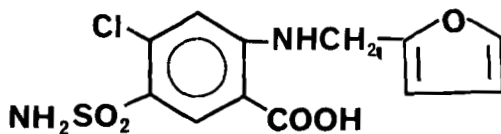
Furosemide is obtained by treating 2-amino, 4-chloro-5-sulfamoyl benzoate ester with furfuryl alcohol.



2-Amino-4-chloro-5-sulfamoyl  
benzoate ester.

Furfuryl alcohol

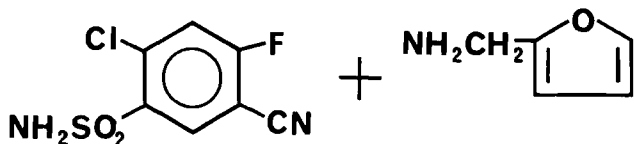
↓ Refluxing for  
2 hrs.



Furosemide

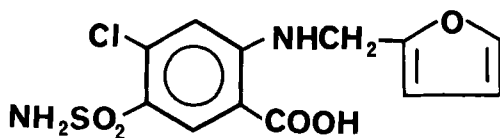
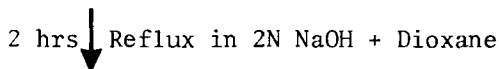
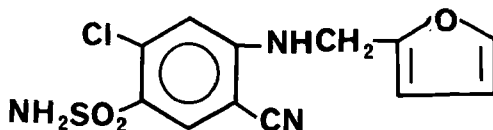
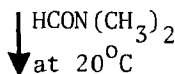
Route 8 (15)

The furosemide is prepared by alkaline saponification of corresponding nitrile which is obtained by the treatment of 3-sulfamoyl-4-chloro-6-chloro cyanobenzene, with furfurylamine.



3-Sulfamoyl-4-chloro-6-chloro-  
cyanobenzene

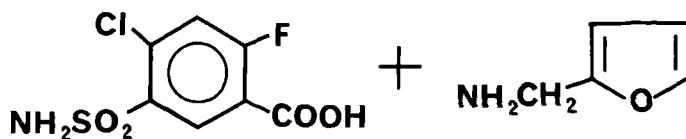
Furfuryl amine



Furosemide

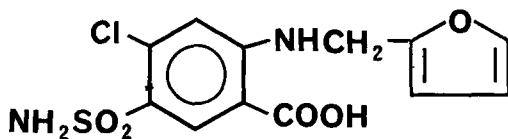
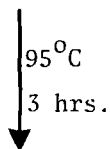
Route 9 (16)

When a mixture of 3-sulfamoyl-4-chloro-6-flourobenzoic acid and furfurylamine is heated for 3 hrs at 95°C.



3-Sulfamoyl-4-chloro-6-flourobenzoic acid

Furfuryl amine

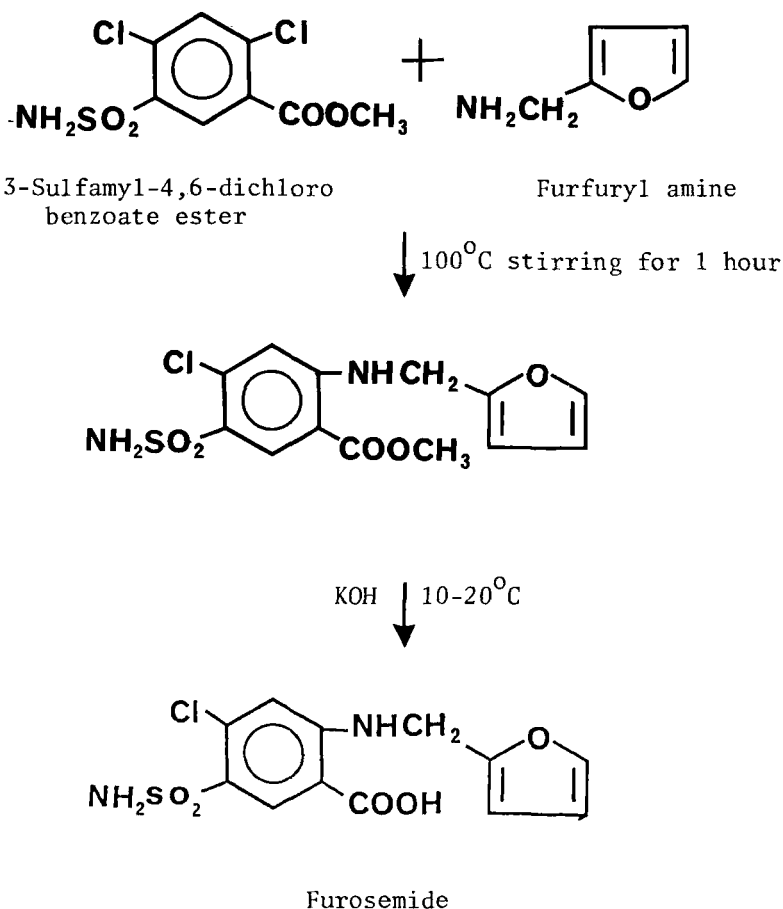


Furosemide



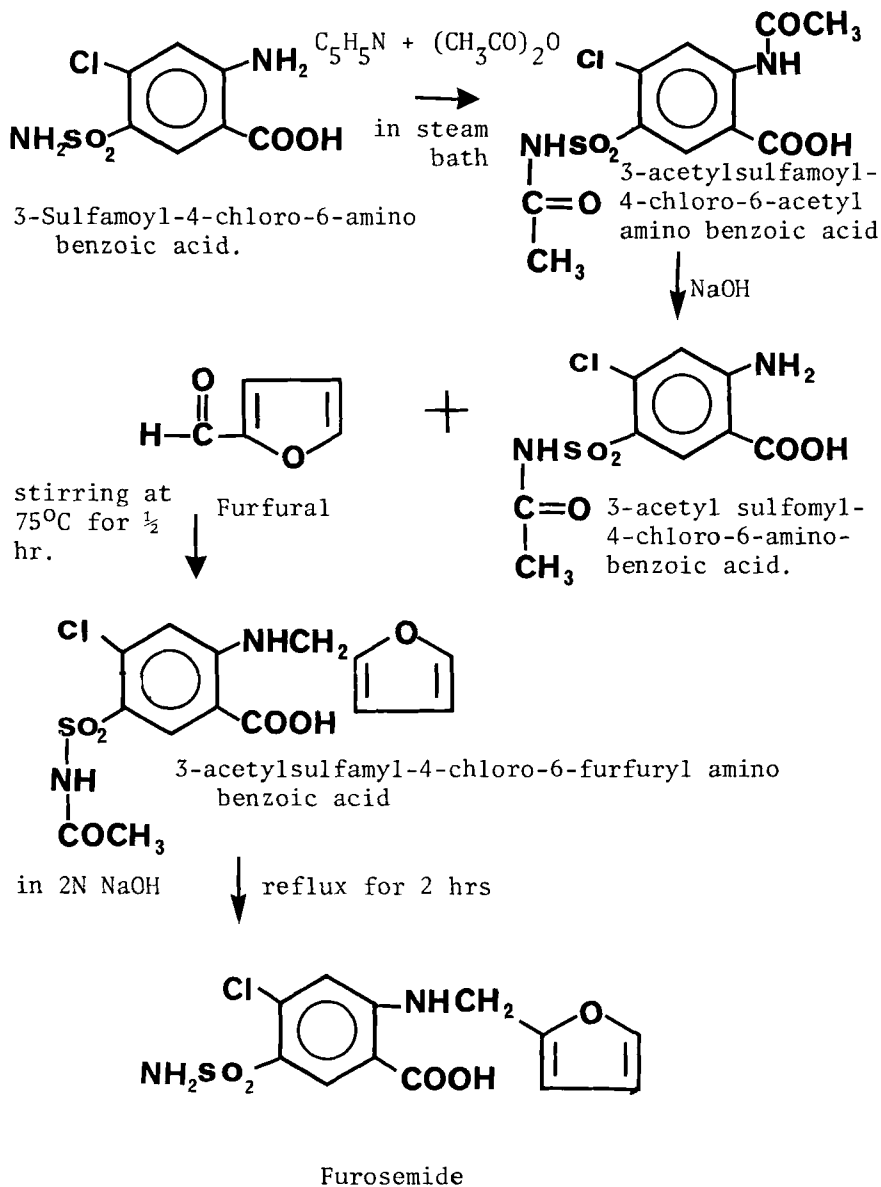
Route 10 (17)

When 3-sulfamoyl-4,6-dichlorobenzoate ester is added with stirring to furfurylamine at room temperature. The mixture is heated at 110°C with stirring for one hour. The obtained mixture is treated with CH<sub>3</sub>COOH to give a crude reaction product. It is then treated with 5 N KOH and stirred for 1 hour at 55°C.



## Route 11

Furosemide is also prepared by the this scheme (18).



#### 4. Absorption and Elimination

Furosemide is absorbed (4) from the gastro-intestinal tract and is strongly bound to plasma proteins. It is mainly excreted in the urine, almost entirely unchanged. In subjects without cardiac or renal diseases, furosemide produced peak plasma concentrations in 30 and 60 minutes after intramuscular and oral administration respectively. About 80% of the dose appeared in the urine within 24 hours after an intramuscular or intravenous injection. In two subjects given furosemide by mouth, 26 and 54% respectively was excreted in urine and 2.1% in faeces. Most of the drug excreted in the urine was passed during the first 4 hours, irrespective of the route of administration.

In an other study (5) the patients with normal renal function, approximately 60% of a single 80 mg oral dose of furosemide was absorbed from the GI tract. When administered to fasting adults in this dosage, the drug appeared in the serum within 10 minutes and reached a peak concentration of 2.3 µg/ml in 60-70 minutes, and was almost completely cleared from the serum in 4 hours. When the same dose was given after a meal, the serum concentration of furosemide increased slowly to a peak of about 1 µg/ml after 2 hours and similar concentrations were present 4 hours after ingestion. However, a similar diuretic response occurred regardless of whether the drug was given with food or to fasting patients. In another study, the rate and extent of absorption varied considerably when 1 g of furosemide was given orally to uremic patients. An average of 76% of a dose was absorbed, and peak plasma concentrations were achieved with 2-9 hours (average 4.4 hours). Serum concentrations required to produce maximum diuresis are not known, and it has been reported that the magnitude of response does not correlate with either the peak or the mean serum concentrations.

The diuretic effect of orally administered furosemide is apparent within 30 minutes to 1 hour and is maximal in the first or second hour. The duration of action is usually 6-8 hours. The maximum hypotensive effect may not be apparent until several days after furosemide

therapy is begun. After IV administration of furosemide, diuresis occurs within 5 minutes, reaches a maximum within 20-60 minutes, and persists for approximately 2 hours. After IM administration, peak plasma concentrations are attained within 30 minutes; onset of diuresis occurs somewhat later than after IV administration. In patients with severely impaired renal function, the diuretic response may be prolonged.

Only limited information is available on the distribution of furosemide. The drug crosses the placenta and is distributed into milk.

Furosemide is approximately 95% bound to plasma proteins in both normal and azotemic patients.

Plasma concentrations of furosemide decline in a biphasic manner. Various investigators have reported a wide range of elimination half-lives for furosemide. In one study (5) the elimination half-life averaged about 30 minutes in healthy patients who received 20-120 mg of drug IV. In another study, the elimination half-life averaged 9.7 hours in patients with advanced renal failure who received 1 g of furosemide intravenously. The elimination half-life was more prolonged in 1 patient with concomitant liver disease.

In patients with normal renal function, a small amount of furosemide is metabolized in the liver to the defurfurylated derivative, 4-chloro-5-sulfamoylanthranilic acid. Furosemide and its metabolite are rapidly excreted in urine by glomerular filtration and by secretion from the proximal tubule. In patients with normal renal function, approximately 50% of an oral dose and 80% of an IV or IM dose are excreted in urine within 24 hours; 69-97% of these amounts is excreted in the first 4 hours. The remainder of the drug is eliminated by nonrenal mechanisms including degradation in the liver and excretion of unchanged drug in the feces. In patients with marked renal impairment without liver disease, nonrenal clearance of furosemide is increased so that up to 98% of the drug is removed from the plasma within 24 hours. One patient with uremia and hepatic cirrhosis eliminated only 58% of an intravenous dose in 24 hours. Furosemide is not removed by hemodialysis.

## 5. Methods of Analysis

### 5.1 Elemental Analysis

$$\text{C}_{12}\text{H}_{11}\text{ClN}_2\text{O}_5\text{S} = 330.77$$

<u>Element</u>	<u>% Theoretical</u>
C	43.57%
H	3.35%
Cl	10.72%
N	8.47%
O	24.19%
S	9.70%

### 5.2 Identification Tests

(i) The light absorption into the range 220 to 330 nm, of the solution obtained in the assay exhibits maximum at 228 nm and 271 nm (2).

(ii) Dissolve about 5 mg in 10 ml of methanol. Transfer 1 ml of this solution to a flask, add 10 ml of dilute hydrochloric acid (1 in 5), and reflux on a steam bath for 15 minutes, cool and add 15 ml of sodium hydroxide test solution and 5 ml of sodium nitrite solution (1 in 1000). Allow the mixture to stand for 3 minutes, add 5 ml of ammonium sulfamate solution (1 in 200) mix and add 5 ml of N-naphthylethylenediamine dihydrochloride solution (1 in 1000). A red to red-violet color is produced (3).

(iii) Dissolve 25 mg in 2.5 ml of alcohol (95%) and add 2 ml of dimethyl aminobenzaldehyde solution, a green colour is produced which becomes deep red. (2)

(iv) Dissolve 25 mg in 25 ml of alcohol (95%) and add 5 ml of water the solution turns blue litmus paper red. (2)

(v) Burns 20 mg by the oxygen flask method, using 5 ml of dilute sodium hydroxide as the absorbing liquid when the process is complete dilute the liquid to 25 ml with water. To 5 ml of the solution so obtained, add 0.1 ml of strong hydroxide solution and 1 ml of 1 N hydrochloric acid, mix and add 0.05 ml of barium chloride solution, a turbidity is produced.

To further 5 ml of the solution obtained as described above add sufficient dilute sulphuric acid and boil gently for 2 minutes add silver nitrate solution yield a white curdy precipitate which is insoluble in  $\text{HNO}_3$  but soluble in dilute ammonia solution.

### 5.3 Titrimetric Method

(i) Dissolve 0.5 g in 40 ml of dimethylformamide and titrate with 0.1 N sodium hydroxide, using bromothymol blue solution as indicator. Repeat the operation without the frusemide, the difference between the titrations represents the sodium hydroxide required. Each ml of sodium hydroxide is equivalent to 0.03308 g of  $\text{C}_{12}\text{H}_{11}\text{ClN}_2\text{O}_5\text{S}$  (2).

(ii) Dissolve about 600 mg of furesemide, accurately weighed in 50 ml of dimethylformamide to which has been added 3 drops of bromothylmol blue and which previously has been neutralized with 0.1 N sodium hydroxide. Titrate with 0.1 N sodium hydroxide to a blue end point. Each ml of 0.1 N sodium hydroxide is equivalent to 33.07 mg  $\text{C}_{12}\text{H}_{11}\text{ClN}_2\text{O}_5\text{S}$  (3).

(iii) The titrimetric method for microdetermination of furosemide in pharmaceutical preparations involves titration of a solution of 1 to 5 mg of drug in  $\sim 1.5$  M HCl with 0.02 M bromosuccinimide, with methyl red as indication. For the cited range, the coefficient of variation is  $< \pm 1\%$  (four determinations at each level), and excipients do not interfere (19).

## 5.4 Spectrophotometric Methods

### 5.4.1 Ultraviolet

A UV method for the assay (20) of frusemide in tablets involves the shaking of powdered sample (containing more than 200 mg of frusemide) with 0.1 N NaOH (50 ml) for one hour, acidify a 10 ml portion with dil. HCl and extract with four 25 ml portions of  $\text{CHCl}_3$  - acetone (4 : 1). Evaporate the combined extracts to dryness, dissolve the residue in methanol, dilute to a concentration of about 5  $\mu\text{g/ml}$  and measure the extinction at 274 nm. The mean recovery is 99.2% with a maximum deviation of  $\pm 1.0\%$ .

In an other method (21) Lasix (furosemide) preparation (0.05 g) was dissolved in  $\text{H}_2\text{O}$ ,  $\text{C}_2\text{H}_5\text{OH}$  or 0.1 NaOH and determined by spectrophotometry at 330, 334, and 335 nm respectively. Furesomide in tablets was determined in  $\text{C}_2\text{H}_5\text{OH}$  at 334 nm and in a 1% ampule solution in  $\text{H}_2\text{O}$  at 330 nm.

Furesomide in tablets is also determined (22) by spectrophotometry at 370 nm with 50%  $\text{C}_2\text{H}_5\text{OH}$  as solvent.

### 5.4.2 Colorimetric Method

The colorimetric method of furosemide involves the production of a blue color when furosemide reacts with butylamine,  $\text{CoCl}_2$  and acetic acid in a medium of anhyd. methanol, the color is measured at 570 nm. Beer's Law is obeyed for 2 to 7 mg of the furosemide in 5 ml of final solution. Anhydrous conditions are essential, and the color is stable for 30 minutes (23).

(i) Furosemide was detected by mixing with a solution of p-dimethylaminocinnamaldehyde in 65%  $\text{H}_2\text{SO}_4$  containing  $\text{FeCl}_3$ , diluting with EtOH, and measuring the absorbance at 530 nm. Beer's Law held for 0.3-1.3 mg of furosemide/10 ml. Mean recoveries from tables were 98.5% for furosemide (24).

### 5.4.3 Nuclear Magnetic Resonance

A quantitative PMR method (25) is described for the analysis of furosemide. The procedure reported

gives accurate results  $98.19 \pm 1.34\%$  and  $99.23 \pm 9.88\%$  for furosemide ampoules and tablets respectively. D<sub>2</sub>O and DMSO-d<sub>6</sub> were used as solvent system and 3-(trimethylsilyl)propionic acid sod. salt as internal standard.

### 5.5 Chromatographic Methods

#### 5.5.1 Column Chromatography (26)

20  $\mu$ l of urine was injected directly on to a stainless-steel column (15 cm X 4.5 mm) packed with LiChrosorb RP-8 or RP-18; various mobile phases; e.g. acetonitrile-phosphate buffer solution (pH 5 to 10) containing tetrabutylammonium hydrogen sulphate, were used. Under optimum conditions furosemide could be separated easily and detected by U.V.

#### 5.5.2 Gas Liquid Chromatography (GLC) (27)

One ml plasma containing 0.3-5.4  $\mu$ g of furosemide was diluted with H<sub>2</sub>O (1:2), acidified and furosemide was extracted with diethyl ether (2 X 5 ml). The combined extracts were evaporated under N<sub>2</sub> and the residue was treated with 0.2 M-NaOH (2 ml). 0.1 M tetrahexylammonium hydrogen sulphate (50  $\mu$ l) and 0.5 M iodomethane in dichloromethane (5 ml). The tube was shaken for 20 minutes at 50°, then 4 ml of organic phase was evaporated at room temperature under N<sub>2</sub>. The residue containing the trimethyl derivative of furosemide was dissolved in hexane (0.2 ml) containing trimethyl furosemide as internal standard, and 5  $\mu$ l of the solution was subjected to g.l.c. on a silanised glass column (1.8 m x 2 mm) packed with 3% of J X R (supelco) on Gas-Chrom Q (100 to 120 mesh) and operated at 245° with a <sup>63</sup>Ni electron-capture detector. The structure of the derivate was confirmed by combined g.l.c. - m.s.

#### 5.5.3 Thin-Layer Chromatography (TLC) (28)

0.5 ml of plasma was treated with methanol (1 ml) and centrifuged. A 50  $\mu$ l aliquot of the supernatant liquid was subjected to t.l.c. on a Kieselgel 60 plate together with standards (0.2 to 10 mg) of furosemide and its metabolite, with CHCl<sub>3</sub>-ethyl



acetate-formic acid (14:6:1) as solvent. The plate was dried and equilibrated with air for 3 h, then sprayed with 10% citric acid in aq. ethanediol (1:1) and immediately scanned with a spectrophotometer in the development direction at 420 nm with excitation at 365 nm. The calibration graph was rectilinear for up to 1 µg of substance per spot. The recovery of added furosemide was 104% (coefficient of variation 4.7%) and of metabolite (4-chloro-5-sulphamoylanthranilic acid) was 86.5% (coeff. of variation 7.2%).

#### 5.5.4 High-Performance Liquid Chromatography (HPLC)

High pressure liquid chromatography HPLC method has wide application for the estimation of furosemide. A summary of variable parameters in a few cases is given in Table (7).

### 6. Acknowledgements

The authors would like to thank Mr. Essam A. Loutfi, Department of Pharmaceutical Chemistry, College of Pharmacy, King Saud University for his technical assistance and Mr. Tanvir A. Butt for typing the manuscript.

Table 7: HPLC methods for the analysis of frusemide.

Stationary phase	Mobile phase	Flow rate	Detection	Remarks	Ref.
Bondapak C <sub>18</sub> corasil (1.8 X 2 mm)	0.02 M KCl-HCl buffer pH 2 con- taining 24% and 26% acetonitrile for urine and plasma samples respectively.	0.9 ml/min	UV at 280	The column was operated at 25°C.	29
μ Bondapak C <sub>18</sub> (10 μm)	Methanol-H <sub>2</sub> O- acetic acid (34: 45:3)	2 ml/min	Fluorimetrically with the use of Corning No. 7-60 primary filter and Kodak No. 2A secondary filter		30
μ Bondapak C <sub>18</sub> reversed phase	50% methanol in containing 0.5% acetic acid	2 ml/min	U.V. 340 nm	Levels of frusemide down to 0.1 μg/ml can be determined	31

Continued (Table 7)

Stationary phase	Mobile phase	Flow rate	Detection	Remarks	Ref.
$\mu$ Bondapack C18 reversed phase	0.05% acetonitrile and phosphoric acid (30:70)		Fluorescent detector with the excitation wavelength 225 nm.	Method needs a little quantity of blood sample i.e (200-300 $\mu$ l)	32
Lichrosorb RP8 (5 $\mu$ )	Methylcyanide-sod. acetate mixture		U.V. detection at 275 nm	The limit of detection is 10 ng/ml.	33
Varian CH-10 reversed phase column	0.5% CH <sub>3</sub> COOH in CH <sub>3</sub> CN-H <sub>2</sub> O (30:70)		Fluorescence detector	N-benzyl-4-chloro-5-sulamoylanthranilic acid as internal standard.	34
$\mu$ Bondapack C18 column (30 cm X 4 mm)	10 mM (NH <sub>4</sub> )HPO <sub>4</sub> in 25% methanol	2 ml/min	U.V. 254 nm	Operated at room temperature	35

Continued (Table 7)

Stationary phase	Mobile phase	Flow rate	Detection	Remarks	Ref.
$\mu$ Bondapack C18	Methanol-0.01 M Na acetate (7:13) pH 5.0	2 ml/min	U.V. at 280 nm	Cephalothin sod. is used as internal standard	36
$\mu$ Bondapack C18	Acetonitrile-0.01 M Na acetate (1:3) pH 5.0	2 ml/min	U.V. at 280 nm	Phenobarbitone sod. is used as internal standard	36
	Acetonitrile-0.5% H <sub>3</sub> PO <sub>4</sub> (3:5)		Fluorimetry at 289 nm		37
Lichrosorb RP-8 (5 $\mu$ m)	Methanol-0.02 M phosphate buffer of pH 3.0 (1:1)	1 ml/min	Fluorimetric detection at 410 nm with excitation at 275 nm.	All procedures are carried out under subdued light	38

Continued (Table 7)

Stationary phase	Mobile phase	Flow rate	Detection	Remarks	Ref.
$\mu$ Bondapack C <sub>18</sub> (30 cm X 4.6 mm)	Acetonitrile- 0.08 M H <sub>3</sub> PO <sub>4</sub> (3:5)		Fluorimetric detection at 389 nm with excitation at 233 nm		39
Radial Pak $\mu$ Bondapack C <sub>18</sub> reversed phase column	30% CH <sub>3</sub> CN (pH 3.5) containing 5 mM sod. hexanesulfo- nate	2.5 ml/min	Fluorescence detection with excitation and emission wave- lengths of 330 and 415 nm.		40
Stainless steel column packed with a pellicu- lar cation exchange resin (305 cm X 1 ml)	50 mM phosphate buffer pH 2.5	0.33 ml/min	Fluorescence detection	Maintain the column tempera- ture at 74°C	41

Continued (Table 7)

Stationary phase	Mobile phase	Flow rate	Detection	Remarks	Ref.
Zorbax ODS (5 $\mu$ m) (15 cm X 4.6 nm)	0.01 M-NaH <sub>2</sub> PO <sub>4</sub> (pH 3.5) - methanol (13:7) for plasma and acetic acid (pH 3.5) - methanol (3:2) for urine	3 ml/min	Fluorimetric detection at 389 nm (exci- tation at 235 nm)		42
$\mu$ Bondapack C <sub>18</sub> (30 cm X 4 mm) equipped with a guard column of C <sub>18</sub> corasil (2 cm X 4 cm)	0.02 M acetate buffer (pH 5.3) containing 22% of acetonitrile	2 ml/min	U.V. at 254 nm		43
Column packed with ODS (5 $\mu$ m)	Methanol-H <sub>2</sub> O- acetic acid (40:57:3)	1.45 ml/min	Fluorescence detection was at 460 to 600 nm (excitation at 330 to 400 nm)	Furosemide was stable in frozen plasma and urine for 113 & 204 days respectively	44

7. References

1. "The Merck Index", 9th ed. Merck and Co., Inc., Rahway, N.J., U.S.A. 1976.
2. "The British Pharmacopoeia" Her Majesty's Stationery Office, London 1973.
3. "The United States Pharmacopoeia" 19th ed., Mack Publishing Company, Easton, PA (1975).
4. "Martindale", The Extra Pharmacopoea" 26th ed. Eds. Norman H. Blacow and Ainky Wade. The Pharmaceutical Press London.
5. "Drug Information" 87. American Society of Hospital Pharmacists. Inc. 4630 Montgomery Avenue. Bethesda MD 20814.
6. Shin, Whanchal; Jeon, Geoun Soo. Proc. Coll. Nature Sci., SNU, 8(1) 45-51 (1983).
7. Unpublished data, Mohammad Saleem Mian (1988).
  - 7a. Neelofur Abdul Aziz Mian, College of Medical Sciences, King Saud University, Riyadh, Private Communication (1988).
  - 7b. Mohammad Saleem Mian, College of Pharmacy, King Saud University. Unpublished data (1988).
  - 7c. Mohammad Saleem Mian, College of Pharmacy, King Saud University. Unpublished data (1988).
  - 7d. Mohammad Saleem Mian, College of Pharmacy, King Saud University. Unpublished data (1988).
8. Foldi, Zoltan, Palusi, Endre, Szinneyei, Eva, Rago, Mrs. Dezso. Hung Telizes 2511 (Cl.C07cd), 08 Aug. 1971.
9. Avidon, V.V. Pleshakov, M.G. Khim. Farm. Zh. 3(3), 25 1969 (Russ).
10. Yellin, Haim, Konjino, Eliezer, U.S. 3,780,067, (Cl.260-347.2; c 07d), 18 Dec. 1973 Appl 139.251 30 Apr 1971.

11. Kovar, K.A., Wojtovicz, G.P. Auterhoff, H. Arch. Pharm. (Weinheim Ger) 307(9), 657-62 1974 (Ger).
12. Christensen, Svend, A. Ger. Offen, 2,043, 439 (cl. C 07cd) 19 May 1971 Brit. Appl 05 Sep. 1939.
13. Christensen, Svend, A. Ger. Offer. 1,801,778 (cl.C07d) 08 May 1969, Brit. Appl. 10 Oct 1967, 6 pp.
14. Christensen, Svend, Age, Ger Offer. 2,206,424 (cl.C.07d A 61K) 24 Aug 1972, Brit. Appl. 4672/71 15 Feb. 1971 9 app.
15. Farbwerke Hoechst A.G.Fr. 1,584,918 (cl.C 07d), 02 Jan 1970 Swiss Appl 08 Jul 1968-12 Sep 1968 11 pp.
16. Ferbwerke Hoechst, A.G. Frd. 1,396,621 (Cl A 61K, c 07c,d) April 23, 1965, Ger Appl April 13, 1963 13 pp.
17. Farbwerke Hoechst, A.G. Ger 1,174,797(cl.C.07d) July 30 1964, App Oct 16, 1962, 3 pp.
18. Farbwerke Hoechst, A.G. Neth. Appl. 6 607,737 (Cl.C 07c) Dec. 6, 1966 Ger. Appl June 5 Sept. 24 Nov. 11, 1965, and May 4, 1966, 20 pp.
19. Shrirama, Miss, Pathak, Y.N.; and Shukla, Chem. Anal. (Klarsaw), 25(1), 91-94 (1980).
20. E.F. Salim, A. Haussler and J.B. Vaughan, J. Pharm. Sci., 57(4), 640-641 (1968).
21. Buryak V.P. Farm. Zh. (Kiev). 6, 55-6, 1976 (Ukrain).
22. Lilo, O. Guerello and Jose Dobrecky, Revta Farm., 111(1-2), 13-16 (1969) (Spanish).
23. Dessouky,k Y.M. and Gad el Rub C.N. Pharm. klee kb) Sci, Ed. 1980, 2 (4), 1128-1130.
24. Moussa Bahia, A. El Kousy Nagalaa, M. Egypt, J. Pharm. Sci., 24(1-4), 21-8 Eng. 1983
25. Hassan Y. Aboul-Enein, A.A. Al-Badr, M. Salah El-Din Rashed, Spectroscopy Letters, 12(4), 323-31 (1979).



26. Lagerstrom Per-Olof. J. Chromatogr. 225(2); Biomed. Apple, 14(2) 476-481, 1981.
27. Lindstroem, B. and Molander, M. J. Chromat. 101(1), 219-221 (1974).
28. Schaefer, Monika; Geissler, Heinrich, E. and Mutschler, Ernst. J. Chromatogr. 143(6), Biomed, Apple 1(6), 636-639 (1977).
29. Lindstroem, B. J. Chromat., 100(1), 189-191 (1974).
30. Carr, Keith; Rane, Anders; and Froelich, Juergen, C. J. Chromatography, 145(3); 421-427 (1978).
31. Roseboom, H., Sorel, R.H.A. Anal. Lett., B11(6), 469-75.
32. Nation, Roger L.; Peng, Geoffrey W., Chiou, Wing, L. J. Chromat., 162(1), 88-93 (1979).
33. Broquaire, M., Mitchard, M. Feuill. Biol., 106, 99-103 (1979).
34. Swezey, Sarah, E.; Meffin, Peter, J., Blaschke, Terrence F., J. Chromatogr., 174(2), 469-73 (1979).
35. Ghanekar, A.G.; Das Gupta, V. and Gibbs, Charles W., jun. J. Pharm. Sci., 67(6), 808-811 (1978).
36. Lin, Emil, T.; Smith, David, E.; Benet, Leslie, Z. and Hoener, Betty-Ann. J. Chromatogr., 163(3) 315-321 (1979).
37. Rapaka, Rao, S.; Roth, Jeri; Goehl, Thomas, J.; and Prasad, vadlamani, K. Clin. Chem., 27(8), 1470-1471 (1981).
38. Kerremans, A.L.M.; Tan, Y.; Van Ginneken, C.A.M. and Gribnav, F.W.J. J. Chromatogr., 229(1), Biomed. Appl., 18(1), 129-139 (1982).
39. Rapaka, Rao, S., Roth, Jeri; Viswanathan, C.; Goehl, Thomas, J., Prasad, Vadlamani, K. and Cabana, Bernard, E. J. Chromatogr., 227(2), Biomed. Appl. 16(2), 463-469 (1982).

40. Kubo, Hiroaki, Li, Haozhi, Kobayashi, Yoshie; Kinoshita, Joshio. Bunseki Kagaku, 35(3), 259-62 (1986).
41. Blair, Andrew D., Forrey, Arden, W.; Meijsen, B. Teri; and Cutler, Ralph E. J. Pharm. Sci., 64(8), 1334-1339 (1975).
42. Lovett, L.J., Nygard, G.; Dura, P., Khalil, S.K.W. J. Liq. Chromatogr., 8(9), 1611-1628 (July, 1985).
43. Guermouche, S., Guermouche, M.H., Mansouri, M., Abed, L. J. Pharm. Biomed. Anal., 3(5) 453-458 (1985).
44. Bauza, M.T.; Lesser, C.L.; Johnston, J.T.; Smith, R.V. J. Pharm. Biomed. Anal., 3(5), 459-467 (1985).

## ANALYTICAL PROFILE OF NALORPHINE HYDROBROMIDE

By

- A) Dr. Muhammad Uppal Zubair  
Assistant Professor,  
Center for University Women Students, King Saud  
University, Riyadh, Saudi Arabia.
  
- B) Mohammad Saleem Mian  
Assistant Researcher,  
Department of Pharmaceutical Chemistry, College of  
Pharmacy, King Saud University, Riyadh, Saudi Arabia.
  
- C) Neelofur Abdul Aziz Mian  
Biochemist,  
Children & Maternity Hospital, Riyadh, Saudi Arabia.

## Contents

### 1. Description

#### 1.1. Nomenclature

##### 1.1.1. Chemical Names

##### 1.1.2. Generic Names

#### 1.2. Formulae

##### 1.2.1. Empirical

##### 1.2.2. Structural

##### 1.2.3. Cas Registry Number

#### 1.3. Molecular Weight

#### 1.4. Elemental Composition

#### 1.5. Appearance, Color, Odor and Taste

### 2. Physical Properties

#### 2.1. Melting Range

#### 2.2. Solubility

#### 2.3. Crystal Structure

#### 2.4. Spectral Properties

##### 2.4.1. Ultraviolet Spectrum

##### 2.4.2. Infrared Spectrum

##### 2.4.3. Nuclear Magnetic Resonance Spectrum

###### 2.4.3.1. $^1\text{H}$ -NMR Spectrum

###### 2.4.3.2. $^{13}\text{C}$ -NMR Spectrum

##### 2.4.4. Mass Spectrum

### 3. Synthesis

### 4. Metabolism

### 5. Stereochemistry

### 6. Thermal Analysis

### 7. Methods of Analysis

#### 7.1. Nalorphine Hydrobromide

##### 7.1.1. Identification

##### 7.1.2. Titrimetric Method

#### 7.2. Nalorphine Hydrochloride

##### 7.2.1. Colorimetric

##### 7.2.2. Spectrophotometric

##### 7.2.3. Thin-Layer Chromatography

## 1. Description

### 1.1. Nomenclature

#### 1.1.1. Chemical Names

- (a) N-Allylnormorphine hydrobromide (1,2).
- (b) N-Allyl-7,8-dehydro-4,5-epoxy-3,6-dihydroxymorphinan hydrobromide (2).
- (c) 7,8-Didehydro-4,5 $\alpha$ -epoxy-17-(2-propenyl)morphinan-3,6 $\alpha$ -diol hydrobromide (3).
- (d) 17-Allyl-7,8-didehydro-4,5 $\alpha$ -epoxymorphinan-3,6 $\alpha$  hydrobromide (3).

#### 1.1.2. Generic Names

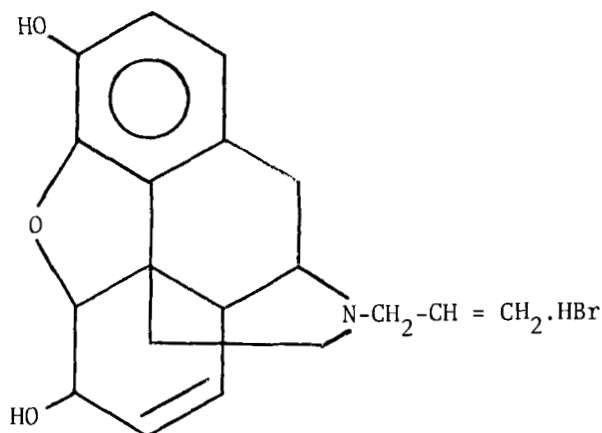
Allorphine, Anarcon, Antorphine, Lethidrone, Nalline, Norfin (4).

### 1.2. Formulae

#### 1.2.1. Empirical

$C_{19}H_{22}BrNO_3$  (1).

#### 1.2.2. Structural



### 1.2.3. CAS Registry

1041-90-3 (nalorphine hydrobromide) (2).

57-29-4 (nalorphine hydrochloride) (2).

62-67-9 (nalorphine) (2).

### 1.3. Molecular Weight

$C_{19}H_{21}NO_3HBr = 392.3$  (2).

### 1.4. Elemental Composition

C, 58.16%; H, 5.66%; Br 20.36%; N, 3.59%, O, 12.23%.

### 1.5. Appearance, Colour, Odor and Taste

An odorless, white to creamy white crystalline powder (2).

## 2. Physical Properties

### 2.1. Melting Range

About  $260^{\circ}$  with decomposition (2).  $258^{\circ}$ - $259^{\circ}$  with decomposition (3).

### 2.2. Solubility

It is soluble in 24 parts of water and in 35 parts of 95% ethanol (1). Aqueous solutions may deposit crystals of the dihydrate. The hydrated form is readily soluble in dehydrated ethanol, and the solutions rapidly give rise to a deposit of the anhydrous form (1).

### 2.3. Crystal Structure

Y.G. Gelders et. al. (5) have calculated and reported crystal data derived from Weissenberg photographs and single crystal diffractometry: (Cu  $K\alpha$ ,  $\lambda = 1.54178 \text{ \AA}$ )  $C_{19}H_{22}BrNO_3$ , Mr = 392.30, orthorhombic, space group  $P2_12_12_1$ ,  $a = 9.287(1)$ ,  $b = 11.749(1)$ ,  $c = 16.249(1) \text{ \AA}$ ,  $V = 1773 \text{ \AA}^3$ ,  $Z = 4$ ,  $D_m = 1.48$ ,  $D_c = 1.47 \text{ Mg m}^{-3}$ ,  $F(000) = 808$ ,  $\mu(\text{Cu } K\alpha) = 36.5 \text{ cm}^{-1}$ .

They also measured (5) intensities of reflections on a Nonius-CAD-4 diffractometer with graphite monochromated copper  $K\alpha$  radiation and a  $\theta$ - $2\theta$  scan.

Table 1: Thermal parameters  $B_{eq} (\text{\AA}^2)$  and atomic co-ordinates ( $\times 10^4$ ) with e.s.d.'s in parentheses for the refined parameters

Atom	<u>x</u>	<u>x</u>	<u>z</u>	<u>B(<math>\text{\AA}^2</math>)</u>
Br	8125(1)	1501(1)	1494(1)	5.52(2)
N	10395(8)	4475(5)	484(4)	3.86(18)
O(1)	3565(6)	6659(7)	1761(4)	6.23(21)
O(2)	5852(7)	7192(5)	635(3)	4.76(17)
O(3)	7113(6)	9269(4)	630(4)	4.44(16)
C(1)	6546(13)	4714(8)	2415(5)	5.02(27)
C(2)	5163(10)	5239(8)	2360(6)	5.13(28)
C(3)	4849(10)	6052(9)	1798(6)	4.83(26)
C(4)	5874(9)	6276(7)	1206(5)	3.90(21)
C(5)	7371(8)	7282(6)	297(4)	3.24(19)
C(6)	8024(9)	8339(6)	649(4)	3.43(19)
C(7)	8768(9)	8174(6)	1463(5)	3.98(21)
C(8)	9534(9)	7249(6)	1599(5)	4.14(22)
C(9)	10140(8)	5187(6)	1258(4)	3.19(19)
C(10)	9189(10)	4618(7)	1889(5)	3.93(22)
C(11)	7599(9)	4965(6)	1819(4)	3.45(19)
C(12)	7197(7)	5692(6)	1208(4)	3.03(17)
C(13)	8143(9)	6174(5)	542(4)	2.91(17)
C(14)	9650(7)	6339(6)	938(4)	2.93(18)
C(15)	8316(10)	5383(7)	-198(4)	3.87(21)
C(16)	9016(9)	4258(8)	18(5)	4.11(23)
C(17)	11155(10)	3376(7)	612(5)	4.51(24)
C(18)	12620(11)	3516(8)	1030(6)	5.39(28)
C(19)	13027(15)	2853(10)	1607(8)	7.64(40)
H(20)	11069	4904	113	3.71
H(01)	3213	6999	2292	5.99
H(03)	7500	10000	625	4.33
H(1)	6827	4165	2917	5.04
H(2)	4348	4969	2764	5.18
H(5)	7394	7354	-350	3.07

The atomic numbering system and the bond lengths of nalorphine hydrobromide have also been calculated (5) and are shown in Figure 1.

It has been reported (5) that most of the bond lengths and bond angles of nalorphine hydrobromide are within the expected limits, however, the bond C(5)-O(2) has been found to be enlarged, probably because of the strain it experiences while linking rings A, C and D as shown in Table II.

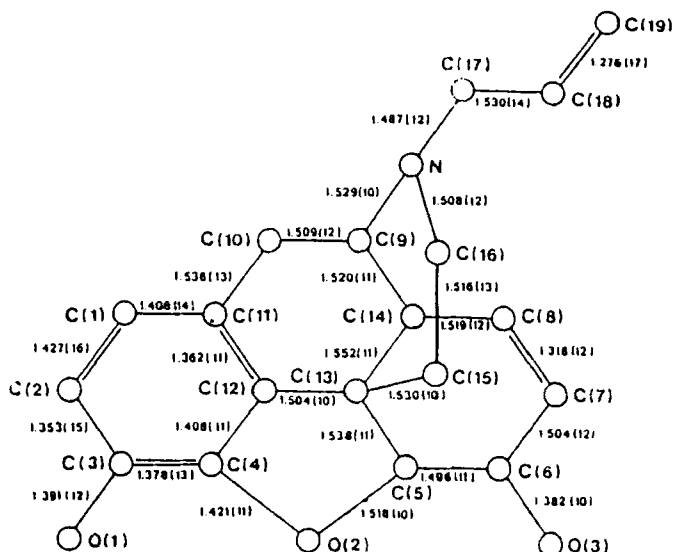


Figure I. Showing bond lengths and atomic numbering scheme of nalorphine hydrobromide (5).



Table II: Bond angles ( $^{\circ}$ ).

<u>Angle</u>		<u>Angle</u>	
C(9)-N-C(16)	111.9(6)	C(10)-C(9)-C(14)	116.9(6)
C(9)-N-C(17)	115.7(6)	C(9)-C(10)-C(11)	113.2(7)
C(16)-N-C(17)	109.0(7)	C(1)-C(11)-C(10)	124.2(8)
C(4)-O(2)-C(5)	106.0(6)	C(1)-C(11)-C(12)	116.2(8)
C(2)-C(1)-C(11)	119.5(8)	C(10)-C(11)-C(12)	119.0(7)
C(1)-C(2)-C(3)	122.8(9)	C(4)-C(12)-C(11)	123.2(7)
O(1)-C(3)-C(2)	125.1(9)	C(4)-C(12)-C(13)	109.0(7)
O(1)-C(3)-C(4)	117.6(9)	C(11)-C(12)-C(13)	126.9(7)
C(2)-C(3)-C(4)	117.2(9)	C(5)-C(13)-C(12)	103.5(6)
O(2)-C(4)-C(3)	126.2(8)	C(5)-C(13)-C(14)	115.0(6)
O(2)-C(4)-C(12)	112.5(7)	C(5)-C(13)-C(15)	111.0(6)
C(3)-C(4)-C(12)	120.5(8)	C(12)-C(13)=C(14)	106.0(6)
O(2)-C(5)-C(6)	107.2(6)	C(12)-C(13)-C(15)	113.5(6)
O(2)-C(5)-C(13)	106.3(6)	C(14)-C(13)-C(15)	107.9(7)
C(6)-C(5)-C(13)	114.4(6)	C(8)-C(14)-C(9)	114.0(6)
O(3)-C(6)-C(5)	113.5(7)	C(8)-C(14)-C(13)	108.5(6)
O(3)-C(6)-C(7)	113.7(6)	C(9)-C(14)-C(13)	107.5(6)
C(5)-C(6)-C(7)	114.6(6)	C(13)-C(15)-C(16)	113.2(7)
C(6)-C(7)-C(8)	120.1(8)	N-C(16)-C(15)	109.5(7)
C(7)-C(8)-C(14)	120.0(8)	N-C(17)-C(18)	113.0(7)
N-C(9)-C(10)	114.0(6)	C(17)-C(18)-C(19)	121.6(10)
N-N(9)-C(14)	104.6(6)		

Solid state conformation of nalorphine hydrobromide, morphine and naloxone has also been compared by measuring their torsion angles (5). An Ortep stereopair is also shown in Fig. II. (5).

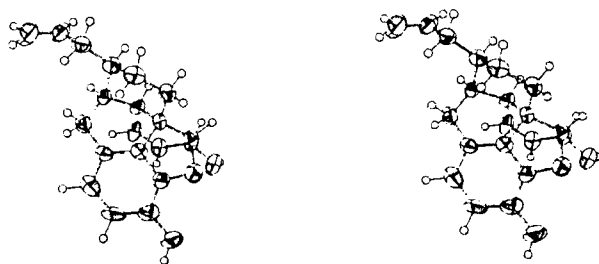


Figure II: Ortep stereopair of nalorphine HBr.

While linkage of nalorphine molecules by hydrogen bond between Br, O and N atoms, along with their values are given in Table III. (5)

Table III: Hydrogen bonding distances ( $\text{\AA}$ ) and angles ( $^\circ$ )

<u>D-H...A</u>	<u>Angle</u>	<u>D-H</u>	<u>H...A</u>	<u>D...A</u>
O(1)-H(01)....Br	140.8	1.00	2.40	3.246(7)
O(3)-H(03)....Br	142.0	0.93	2.33	3.119(6)
N-H(20)...O(3)	173.5	1.00	1.83	2.829(10)

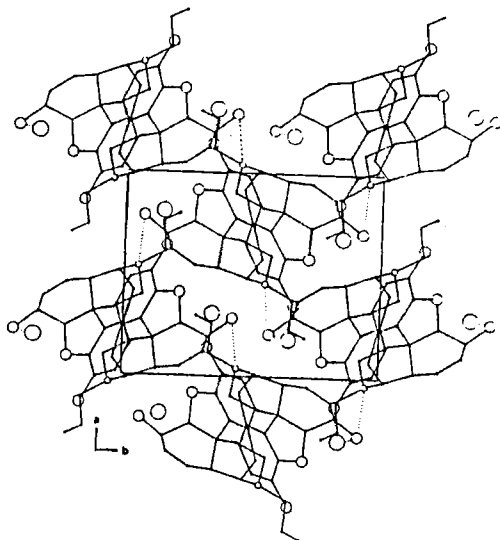


Figure III. Projection in the (001) plane of nalorphine HBr structure showing the packing and the hydrogen-bond scheme. Large, medium and small circles represent Br, O and N atoms respectively (5).

## 2.4. Spectral Properties

### 2.4.1. Ultraviolet Spectrum

The UV spectrum of nalorphine hydrobromide (Fig. IV) was scanned from 200 to 350 nm, using Pye Unicam PU 8800 spectrophotometer (6). The UV spectrum of 0.01 percent solution in ethanol shows a maximum at 285 nm (1).

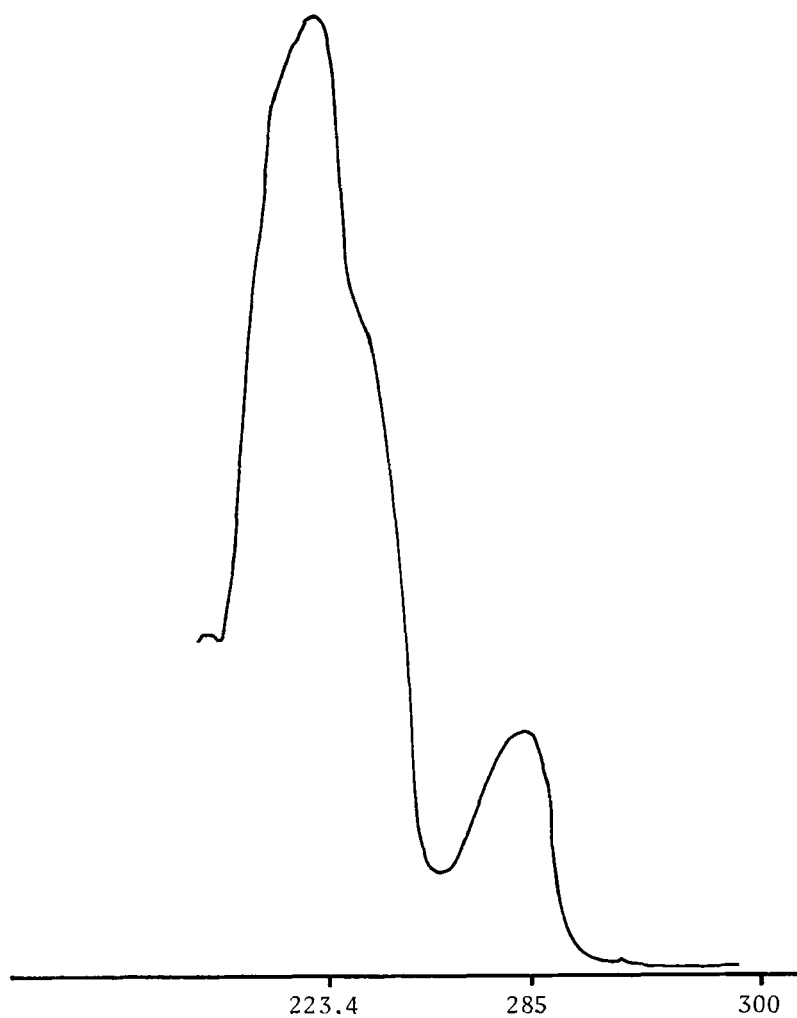


Figure IV. The UV spectrum of nalorphine hydrobromide in ethanol.

### 2.4.2. Infrared Spectrum

The infrared spectrum of nalorphine hydrobromide as KBr disc is shown in Fig. V. The spectrum was scanned with an F.T.I.R., 1500 model, Perkin Elmer spectrophotometer (7). Principal absorption bands are at 3360, 2940, 1635, 1610, 1500, 1460, 1300, 1160, 1120, 960, 945 and 785  $\text{cm}^{-1}$ .

### 2.4.3. Nuclear Magnetic Resonance Spectra

#### 2.4.3.1. $^1\text{H}$ -NMR spectrum

The PMR spectrum of nalorphine hydrobromide (8) in deuterated dimethylsulfoxide was recorded on a Varian T60A 60 MHz NMR spectrometer, using tetramethylsilane (TMS) as an internal standard (Fig. VI). The following structural assignments have been made.

Table IV: PMR spectrum characteristics of nalorphine hydrobromide

<u>Chemical shift (<math>\delta</math>) ppm</u>	<u>Assignment</u>
6.50	H-1, H-2 (aromatic)
4.7 -4.86	H-5
5.26-5.62	$\text{CH} = \text{CH}_2$ (m)
1.9 -4.40	7, 8, 9, 10, 15, 16- $\text{CH}_2$

#### 2.4.3.2. $^{13}\text{C}$ -NMR Spectrum

The  $^{13}\text{C}$ -NMR spectra of nalorphine and nalorphine HCl has been reported (9) and is given in Table V.

### 2.4.4. Mass Spectrum

The mass spectrum of nalorphine has been reported (10) and is shown in Fig. VII.

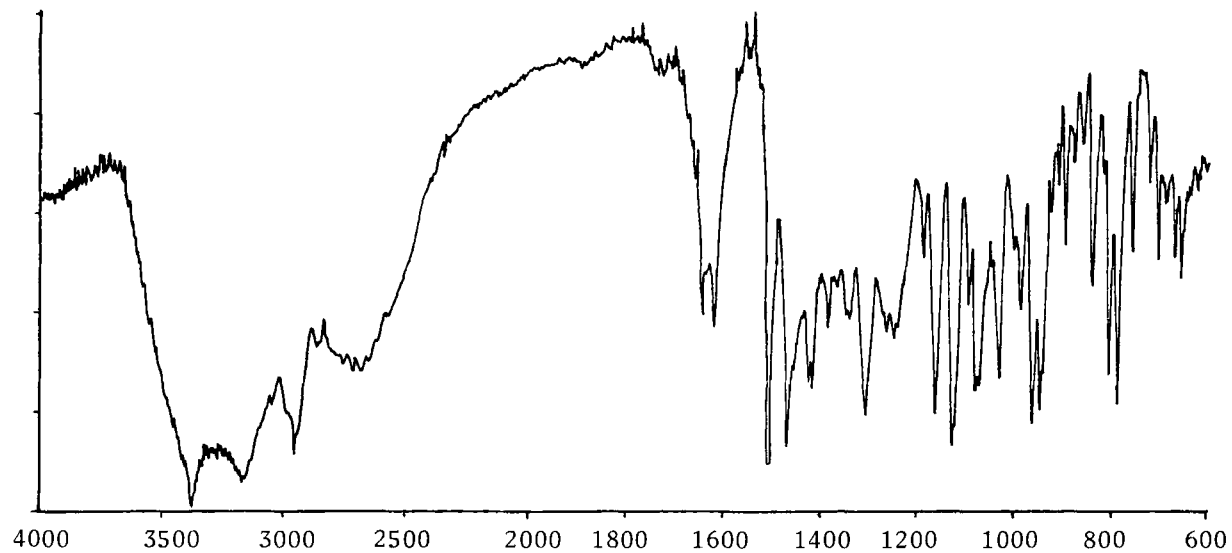


Figure V. Infrared spectrum of nalorphine hydrobromide as KBr disc.

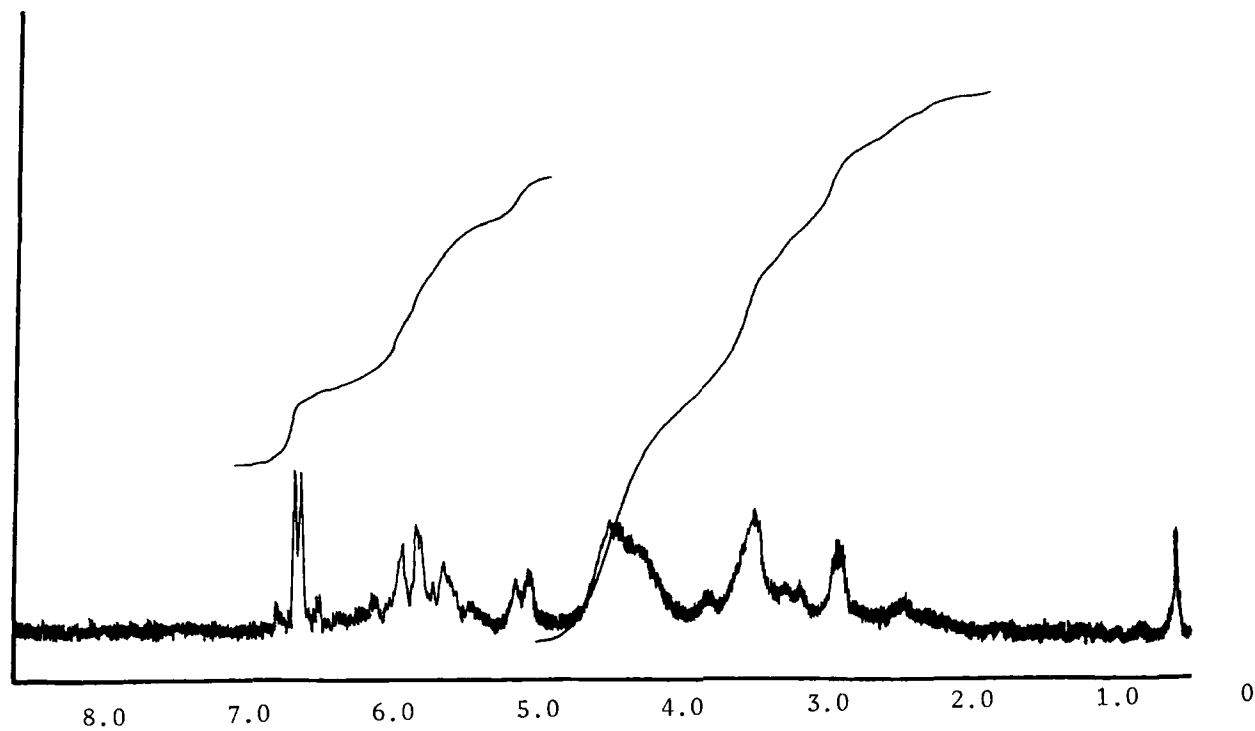


Figure VI. PMR spectrum of nalorphine hydrobromide in DMSO-d<sub>6</sub>.

Table V:  $^{13}\text{C}$  NMR spectra of nalorphine HCl in  $\text{D}_2\text{O}$  - TFA (80:20), and of nalorphine in  $\text{DMSO-d}_6$ .

<u>Carbon No.</u>	<u>Chemical shift (<math>\delta</math>) ppm</u>	
	<u>Nalorphine HCl</u>	<u>Nalorphine</u>
C-1	121.0	118.5
C-2	118.1	116.4
C-3	138.1	138.5
C-4	165.1	146.2
C-5	90.1	9.5
C-6	66.0	66.4
C-7	133.1	133.4
C-8	124.9	128.4
C-9	58.5	56.0
C-10	20.8	21.1
C-11	122.6	125.4
C-12	128.8	131.0
C-13	42.2	43.5
C-14	38.5	40.5
C-15	32.4	35.4
C-16	45.8	44.0
C-17	57.2	57.6
C-18	124.9	136.4
C-19	126.8	117.0

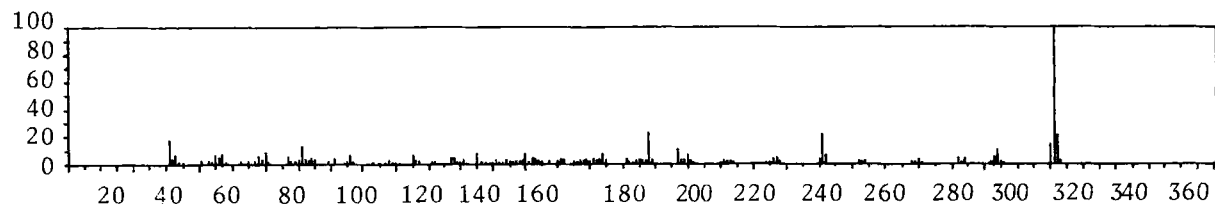
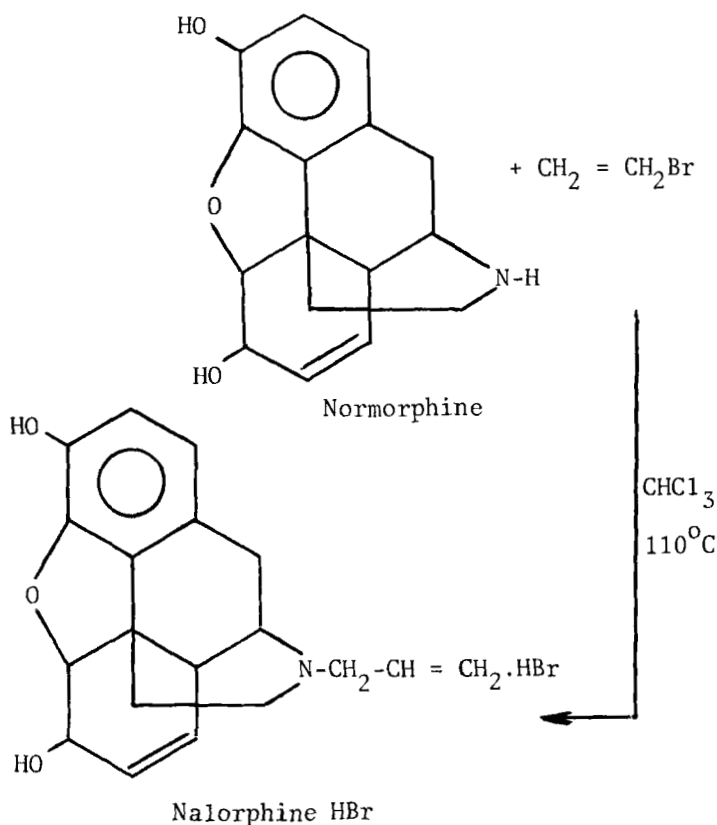


Fig. VII. Mass spectrum of nalorphine.

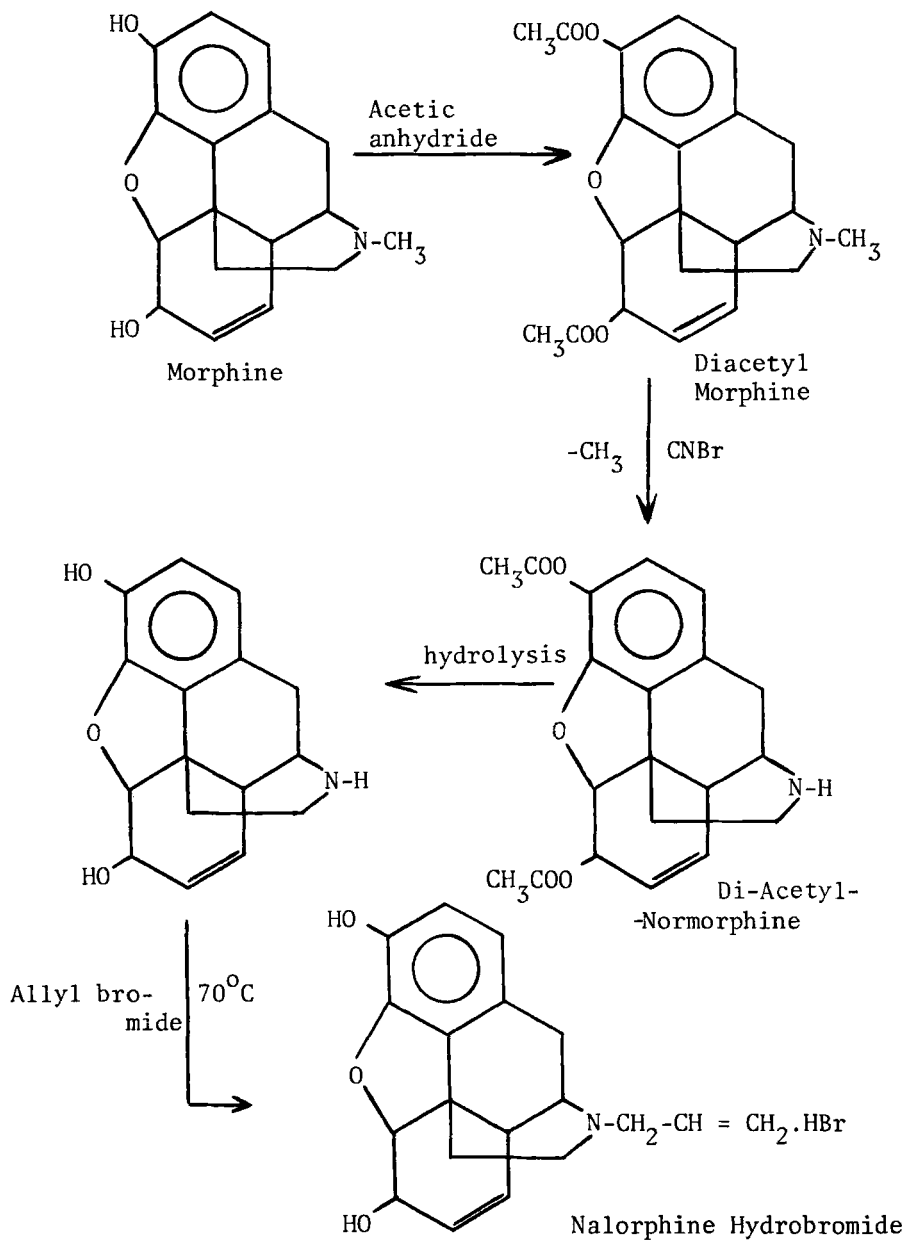


3. SynthesisScheme I (11,12)

Normorphine has been reported to react readily with allyl bromide in chloroform at  $110^{\circ}\text{C}$ . Its structure was confirmed by converting it into N-allyl norcodein, according to Rodionov's method.

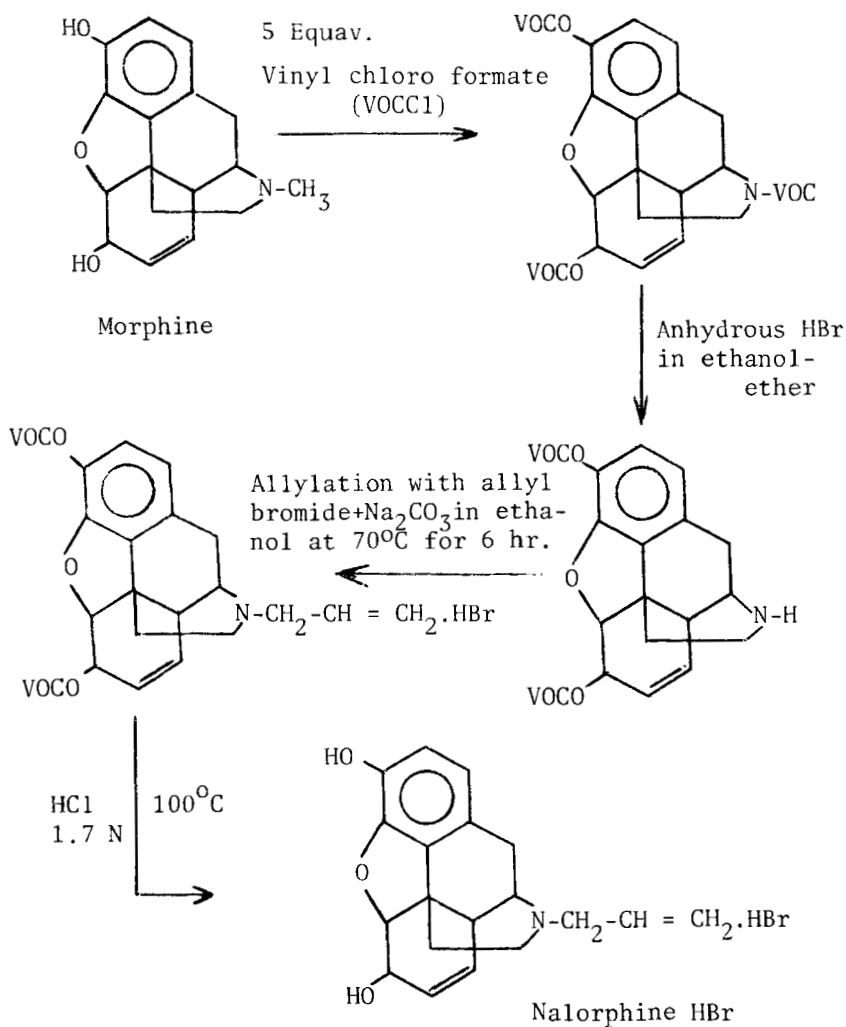
Scheme II (13-16)

Initially both the hydroxyl groups of morphine are acetylated with acetic anhydride. It is then reacted with cyanogen bromide to remove the methyl group and convert it to normorphine, which on reaction with allylbromide at  $70^{\circ}\text{C}$  is converted to allylnormorphine hydrobromide.

Scheme II

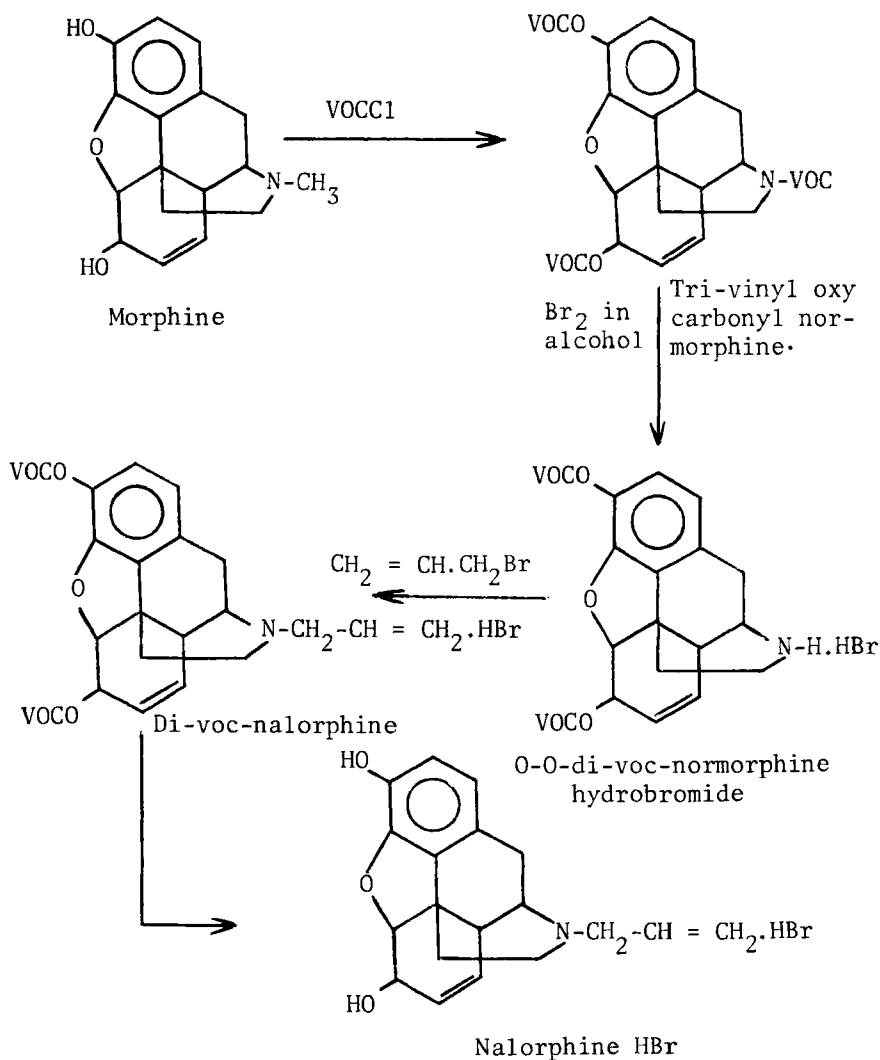
## Scheme III

Morphine is reacted with  $\text{VOCCl}$  in dichloroethane to afford 3  $\text{VOCl}$  derivative. Selective N- $\text{VOC}$  removal was effected with anhydrous hydrogen bromide in ethanol-ether at  $25^\circ\text{C}$ . N-allylation was achieved with allyl bromide and sodium carbonate in ethanol at  $70^\circ\text{C}$  for 6 hrs. The removal of  $\text{VOCl}$  groups was effected with 1.7-N  $\text{HCl}$  to yield nalorphine hydrobromide (15).



## Scheme IV

Morphine is converted to tri-voc-normorphine with VOCCl reagent and its subsequent conversion to di-voc-normorphine is achieved with a mild acid or by titration with  $\text{Br}_2$  and subsequent dilution with alcohol (17). It is then reacted with allyl bromide and subsequent removal of VOC groups affords nalorphine hydrobromide.



#### 4. Metabolism

Nalorphine has been reported to be converted to normorphine by rats (18) and cats (19) in vivo and by rat liver microsomes in vitro (20). It is conjugated in rats (21), dogs (22), rabbits and cats (23). Nalorphine-3-glucuronide and nalorphine 3-ethereal sulphate have also been isolated from the urine of cats and rabbits (23). Recently nalorphine 6-glucuronide has also been isolated from the urine of cats and dogs (24).

Nalorphine has been reported (2) to be poorly absorbed when given orally; however, on parental administration it is absorbed and readily passes into the brain and across the placenta. It is mostly metabolised in the liver and is excreted in the urine. Small amount of unchanged nalorphine is also excreted in urine. Nalorphine hydrobromide is mainly used to antagonise narcotic-induced respiratory depression.

#### 5. Stereochemistry

The numbering is the standard numbering as for morphine (which follows that for phenanthrene). Nalorphine differs structurally from morphine, only by substitution of an allyl group for the N-methyl group. The absolute configuration of nalorphine is believed to be the same as for morphine. A model of nalorphine is shown in Fig. VIII.

#### 6. Thermal Analysis (DSC)

A differential scanning calorimetry curve was obtained (Fig. IX) on a Perkin-Elmer DSC-2C differential calorimeter (25). Nitrogen was used as the purge gas. Scan was performed at a rate of 40°C/min from 80°C to 350°C. The DSC curve revealed an endothermic melting peak (Max 260.19°C), followed by decomposition of the sample.

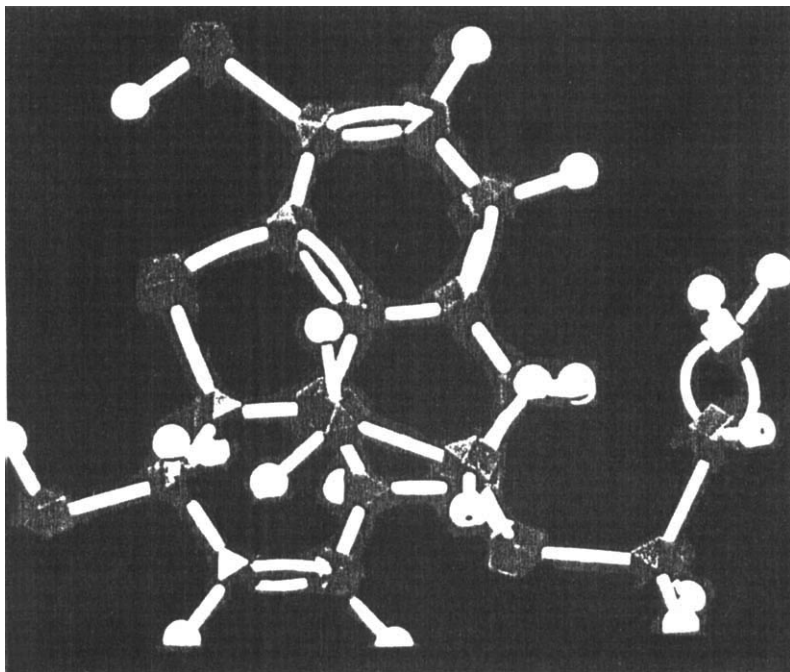


Figure VIII. Atomic model of nalorphine.

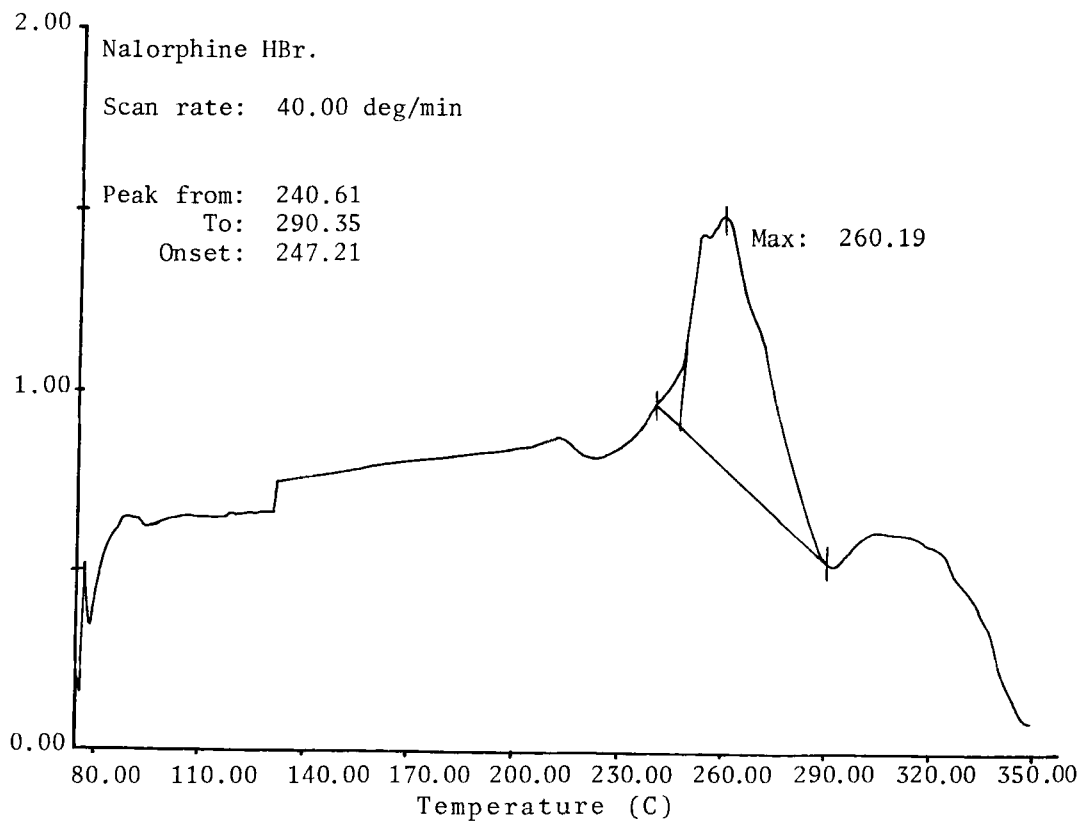


Figure IX. Differential scanning calorimetry curve of nalorphine hydrobromide.

## 7. Methods of Analysis

### 7.1. Nalorphine Hydrobromide

#### 7.1.1. Identification

a) A 0.01 percent w/v solution of nalorphine hydrobromide in a 2-cm wide cell absorbs light from 230 to 350 nm and exhibits a maximum at 285 nm; and its extinction at 285 nm is about 0.78 (1).

b) A 0.01 percent w/v solution, in a 0.1 N sodium hydroxide solution, in 2-cm layer cell exhibits a maximum at 298 nm and extinction at 298 nm is about 1.2 (1).

c) A drop of dilute ammonium hydroxide solution on addition to 5 ml, of a 3 percent solution of nalorphine hydrobromide, immediately produces a bulky white precipitate, which is soluble in sodium hydroxide solution (1).

d) Addition of a drop of ferric chloride solution to 5 ml of a 3 percent w/v solution of nalorphine hydrobromide gives rise to blue color (1).

#### 7.1.2. Titrimetric Method (1)

0.5 g of nalorphine hydrobromide is dissolved in 15 ml of water and 5 ml of dilute ammonia solution is added. Nalorphine is completely extracted into a mixture of three volumes of chloroform and one volume of isopropyl alcohol, washing each extract with the same 10 ml of water. The extracts are combined and the solvent is removed by evaporation. 5 ml of 95% alcohol previously neutralised to methyl red solution is added to the residue, and is later removed by evaporation, and the process is repeated. Then dissolve the residue in 1 ml of the neutralised 95% ethanol and again evaporate. Dissolve the residue in 1 ml of neutralised 95% Alcohol, and 20 ml of 0.1-N hydrochloric acid and 10 ml of water and the mixture is titrated with 0.1 N sodium hydroxide solution, using methyl red solution as indicator. Each ml of 0.1-N hydrochloric acid is equivalent to 0.03923 g of  $C_{19}H_{21}NO_3$ , HBr.



## 7.2. Nalorphine Hydrochloride

### 7.2.1. Colorimetric

Solid nalorphine hydrochloride or a volume of injection equivalent to 0.010 g of nalorphine hydrochloride is diluted with 0.1 M hydrochloric acid to 100 ml. To 15.00 ml of this solution, are added 10 ml of 0.1-M sodium nitrite, and after 10 minutes 5.0 ml of 5-M ammonia solution is added and its volume is made up to 50 ml with water. A standard solution containing 10 mg of nalorphine hydrochloride, and a blank solution, is similarly prepared as described above. The absorbance is measured at 440 nm, and the strength of the unknown solution calculated.

### 7.2.2. Spectrophotometric

Nalorphine hydrochloride as solid or as an injection can be determined spectrophotometrically (26). About 25 mg of nalorphine hydrochloride is accurately weighed and transferred to a 250 ml flask. It is dissolved in water and made up to volume. Its absorbance at 285 nm is measured and compared with a standard sample solution.

### 7.2.3. Thin-Layer Chromatography

Thin-layer chromatography has been applied for the determination of nalorphine hydrochloride (27). 50 g of it were applied to a silica gel G F<sub>254</sub> thin layer plate and eluted in a mixture of, ethanol-dioxane-benzene-ammonia solution 25% (4:8:7:1). The spots were detected in U.V. light (254 nm) and also by spraying with Dragendorff reagent.

## References

1. "The British Pharmacopoeia", Her Majesty's Stationary Office, Cambridge, p. 312 (1973).
2. "Martindale", The Extra Pharmacopoeia", 28th ed., Eds. J.E. Reynolds and A.B. Prasad, p. 1141, The Pharmaceutical Press, London (1982).
3. "Dictionary of Organic Compounds", Fifth ed., p. 4137, Chapman and Hall, 733 Third Avenue, New York
4. "The Merck Index", 10th ed., Merck and Co., Inc., Rahway, N.J., U.S.A. (1983).
5. Y.G. Gelders, C.I. De Ranter and C. Yan Rooijen-Reiss., Cryst. Struct. Comm., 8, 995 (1979).
6. Mohammad Uppal Zubair, Neelofur Abdul Aziz Mian, unpublished data (1988).
7. Mohammad Uppal Zubair, Neelofur Abdul Aziz Mian, unpublished data (1988).
8. Mohammad Uppal Zubair, Mohammad Saleem Mian, unpublished data (1988).
9. Ernest L. Eliel, Morris-Natschke Susan and Vera M. Kolb, Org. Magn. Reson. 22(4), 258-62 (1984).
10. "Registry of Mass Spectral Data", Vol. 3, John Wiley and Sons, New York.
11. John Weijlord and A.E. Erickson, J. Am. Chem. Soc. 64, 869 (1942).
12. Rodionov, Bull. Soc. Chim, 39, 305 (1926).
13. K.W. Bentley, The Chemistry of the Morphine Alkaloids, Oxford Press, London, 1954; G. Stork, The Morphine Alkaloids, Ch 7 in the Alkaloids, Vol. VI, Edited by R.H.F. Monske, Academic Press, New York, N.V., 1960.
14. M.M. Abdel-Monem and P.S. Portoghese, J. Med. Chem., 15, 208 (1972).
15. K.C. Rice, J. Org. Chem., 40, 1950 (1975) and R.F. Olofron and R. Schnur, Tetrahedron Letters No. 18, pp. 1571-1574, 1977.

16. T.A. Montzka, J.D. Matiskella, and R.A. Party Ka, Tetrahedron Lett., 1325 (1974).
17. R.C. Schnur, Ph.D. Thesis, The Pennsylvania State University, 1973.
18. K. Milthers, Acta Pharmacol. Toxicol., 19, 235 (1962).
19. L. Tompier and A. Penna-Herreros, Arch. Biol. Med. Exp., 3, 146 (1966); through Chem. Abstr., 67, 42252x (1967).
20. J. Axelrod and J. Cochin, J. Pharmacol. Exp. Ther., 121, 107 (1957).
21. L.A. Woods and H.E. Muchlenleeck, J. Pharmacol. Exp. Ther., 120, 52 (1957).
22. C.C. Hug, Jr, and L.A. Woods, J. Pharmacol. Exp. Ther., 142, 248 (1963).
23. J.M. Fujimoto, W.M. Watrous and V.B. Harstad, Proc. Soc. Exp. Biol. Med., 130, 546 (1969).
24. S.Y. Yeh and L.A. Woods, J. Pharmaceut. Sci., 60, 148, (1971).
25. Mohammad Uppal Zubair, Neelofur Abdul Aziz Mian, unpublished data (1988).
26. "The United States Pharmacopoeia, 20th Edition, p. 539 (1980).
27. A. Kjaervig Kaistensen, Dansk Tidsskr. Farm., 43, 220-224 (1969).

# NIFEDIPINE

SYED LAIK ALI

**NIFEDIPINE**  
**SYED LAIK ALI**

1. History
2. Nomenclature
3. Description
  - 3.1 Name, Formula, Molecular weight
  - 3.2 Appearance, Colour, Odour
4. Synthesis
5. Physical Properties
  - 5.1 Solubility
  - 5.2 Loss on Drying
  - 5.3 Melting Point
  - 5.4 Sulfates
  - 5.5 Chlorides
  - 5.6 Sulphated Ash, Residue on Ignition
  - 5.7 Heavy Metals
  - 5.8 Dissociation Constant
  - 5.9 Distribution Coefficient
  - 5.10 Light Sensitivity
  - 5.11 Sensitivity to Temperature
  - 5.12 Related Compounds
  - 5.13 Impurities Titrable with Perchloric Acid
  - 5.14 Crystal Structure
  - 5.15 Ultraviolet Spectrum
  - 5.16 Infrared Spectrum
  - 5.17 Nuclear Magnetic Resonance Spectrum
  - 5.18  $^{13}\text{C}$ -NMR-Spectrum
6. Colour and Identification Reactions
7. Stability and Degradation

8. Methods of Analysis
  - 8.1 Titrimetry
  - 8.2 Polarography
  - 8.3 UV Spectrophotometry
  - 8.4 Fluorimetry
  - 8.5 Chromatographic Methods
    - 8.5.1 Thin Layer Chromatography
    - 8.5.2 Gas Liquid Chromatography
    - 8.5.3 High Performance Liquid Chromatography
    - 8.5.4 Gas Chromatography-Mass Spectrometry
9. Radioactive Measurements of C-14 Labelled Nifedipine in Body Fluids
10. In vitro Dissolution
11. Drug Metabolism and Pharmacokinetics
12. Acknowledgements
13. References

## Nifedipine

### 1. History

Dihydropyridine chemistry began in 1882 when Hantzsch (1) first reported them as stable intermediates in the pyridine synthesis that bears his name. In the subsequent 50 years modifications of the original synthesis were developed and some reactions of dihydropyridine were studied. The recent interest in dihydropyridines can be traced to the coenzyme reduced nicotinamide adenine dinucleotide (NADH) and the unique ability of this compound in biological systems to reduce unsaturated functional groups (2,3). In 1949 A.P. Phillips reported on the weak analgetic and curare-like activities of a few dihydropyridine derivatives (4). In search for orally active drugs for the treatment of coronary insufficiency F. Bosset synthesised in 1966 a compound designated Bay a 1040 (5) which, following its introduction as nifedipine (Adalat) in 1975 has since become one of the major cardiovascular drugs (6). The cardiovascular activities of 4-aryl, 1,4-dihydropyridine, 3,5-dicarboxylic acid esters were discovered independently in the sixties in the laboratories of Bayer, Germany (7,8) and Smith, Kline and French (9).

### 2. Nomenclature

1,4-Dihydro-2,6-dimethyl-4-(2-nitrophenyl)-pyridin-3,5-dicarboxylic acid-dimethyl ester; Dimethyl 1,4-dihydro-2,6-dimethyl-4-(o-nitro-phenyl)-3,5-pyridin-dicarboxylate. The formula is illustrated at the next page (Fig. 1).

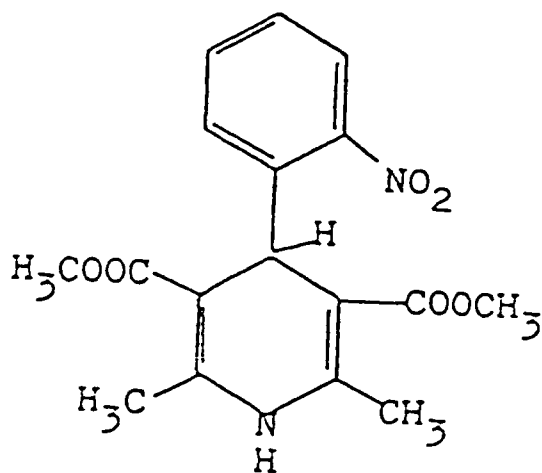


Fig. 1

Nifedipine Structural Formula



### 3. Description

#### 3.1 Name, Formula, Molecular weight

Nifedipine;  $C_{17}H_{18}N_2O_6$ ;  
346.30

#### 3.2 Appearance, Colour, Odour

Yellow, odourless and tasteless crystalline powder, thermostable, non-hygroscopic.

### 4. Synthesis

The preparation of nifedipine is based upon the classical Hantzsch 1,4-dihydropyridine synthesis, i.e. the reaction of 1 mole 2-nitro-benzaldehyde with 2 moles methyl acetoacetate and 1 mole concentrated aqueous ammonia in refluxing methanol which leads to the formation of nifedipine. This reaction can be postulated as the Knoevenagel condensation of 2-nitrobenzaldehyde with methyl acetoacetate and enamine formation from methyl acetoacetate and ammonia as the primary steps. The methyl 2-(2-nitrobenzylidene)-acetoacetate and methyl 3-aminocrotonate thus formed then react to the dihydropyridine in a kind of cyclising Michael addition. This scheme has been realised on a technical scale in the production of nifedipine (6). Crucial for a commercial acceptable synthesis was the development of a new process for the preparation of 2-nitrobenzaldehyde. This new process starts from 2-nitrotoluene which is condensed with diethyl oxalate in the presence of sodium ethoxide-ethanol to give 3-(2-nitrophenyl)-pyruvate

which is halogenated with technical sodium hypochloride and cleaved in a haloform reaction to give 2-nitrobenzylidenchloride in one step. Subsequent hydrolysis with sulfuric acid furnishes 2-nitrobenzaldehyde (6a). This synthesis scheme is illustrated in Fig. 2.

9 g o-nitrobenzaldehyde were dissolved in 15 ml methanol, 16 mg methyl acetoacetate and 6.4 ml 25 % ammonia were added. This mixture was refluxed to boiling for five hours, cooled and left aside overnight in refrigerator. The precipitated yellow crystals of nifedipine were obtained in 82 % yield which were separated and recrystallised (15).

The preparative synthesis of the metabolites follows the well-known rules of dihydropyridine chemistry. Nifedipine is oxidised with nitric acid to the nitropyridine derivative. Selective hydrolysis of one ester group with methanolic potassium hydroxide provides the pyridine-monocarboxylic acid. Several routes are available for the synthesis of the pyridine-lactone compound. One route which possibly simulates the biotransformation involves bromination of a ring methyl group with N-bromosuccinimide, nucleophilic substitution of bromide, acid hydrolysis of the acetyl group with subsequent intramolecular transesterification to give the lactone (6).

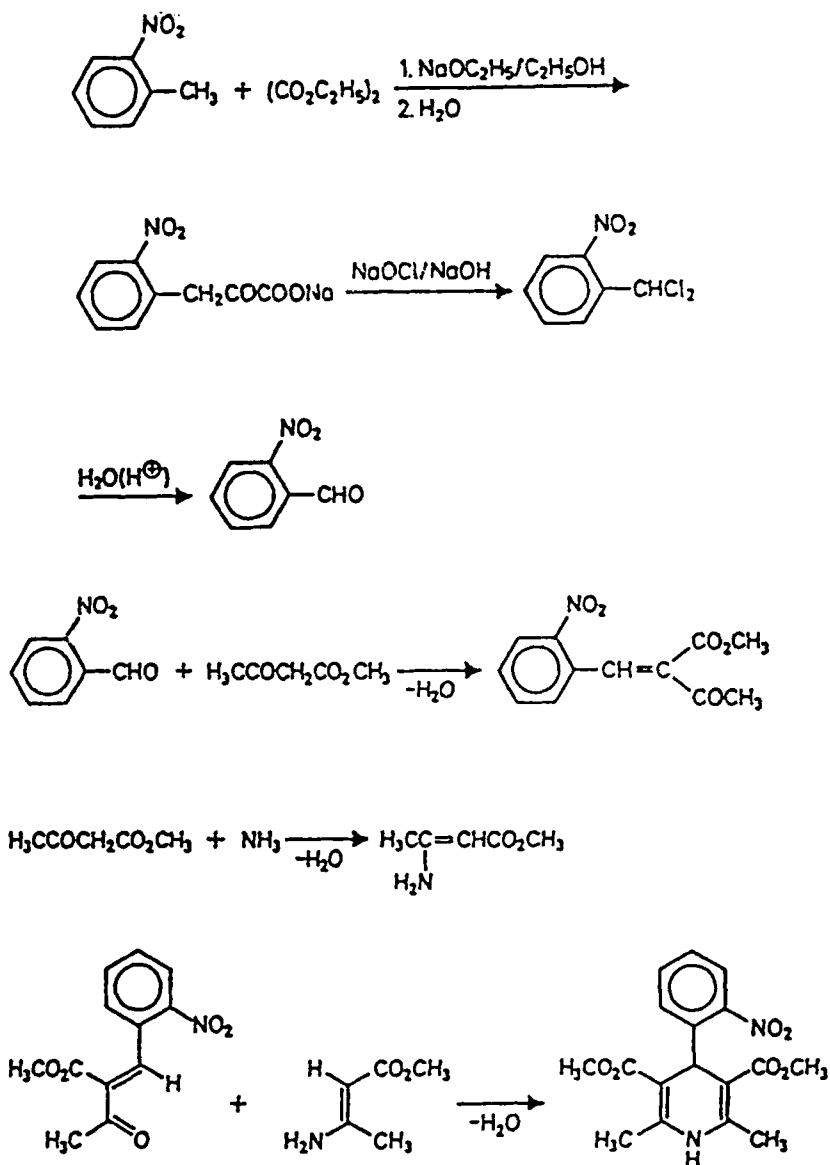


Fig. 2 (6)

Nifedipine Synthesis Scheme

## 5. Physical Properties

### 5.1 Solubility (10)

Nifedipine is freely soluble at 20°C in acetone (250 g/l), in methylene chloride (160 g/l), in chloroform (140 g/l), soluble in ethyl acetate (50 g/l), slightly soluble in methanol (26 g/l) and ethanol (17 g/l) and practically insoluble in water. Further the solubility at 37°C in buffer solutions of different pH-values can be given as: pH 4: 0.0058 g/l; pH 7: 0.0056 g/l; pH 9: 0.0078 g/l; pH 13: 0.006 g/l.

### 5.2 Loss on Drying (11)

It loses not more than 0.5 % of its weight when dried at 105°C to constant weight.

### 5.3 Melting Point (11)

171-175°C

### 5.4 Sulfates (11)

Maximum 500 ppm with limit test for sulfates

### 5.5 Chlorides (11)

Maximum 200 ppm with limit test for chlorides

### 5.6 Sulphated Ash, Residue on Ignition (11)

Not more than 0.1 %, at an ignition temperature of 600°C being used, determined with 1 to 2 g.

### 5.7 Heavy Metals (11)

Maximum 10 ppm

### 5.8 Dissociation Constant (12)

This is determined with tetrabutyl ammonium hydroxide in dimethyl formamide as solvent and a  $\text{PK}_a$ -value (acidic)  $> 13$  is obtained. The  $\text{PK}$  values of calcium antagonists such as nifedipine and verapamil were measured using a potentiometric microtitration technique. Nifedipine is not protonated and must exert its action via the unchanged form (12).  $\text{PK}_a$ -value (basic) is about -0.9. Owing to the extreme low basicity of the dihydropyridine nitrogen it is not possible to obtain stable salts with acids.

### 5.9 Distribution Coefficient (10)

- i) cyclohexane - aqueous buffer solution of any pH-value between 0 and 13 = 95:5
- ii) octanol-water = about 10000:1

### 5.10 Light Sensitivity (10)

The substance is sensitive to light in solid form and extremely sensitive to light in dissolved state in solution.

### 5.11 Sensitivity to Temperature (10)

Should not be stored above 25°C, should be protected from frost.

### 5.12 Related Compounds (11)

- a) Nitropyridine compound [Dimethyl 4-(2-Nitrophenyl)-2,6-dimethyl pyridine-3,5-dicarboxylate]: not more than 0.2 %
- b) Nitrosopyridine compound [Dimethyl 4-(2-Nitroso-phenyl)-2,6-dimethyl pyridine-3,5-dicarboxylate]: not more than 0.2 %

The test is to be performed through thin layer chromatography on silicagel plates with diisopropyl ether as the solvent using corresponding USP reference substances.

### **5.13 Impurities Titrable with Perchloric Acid (11)**

Not more than 0.12 ml of 0.1 N perchloric acid is consumed for each g of nifedipine when titrated with about 4 g of nifedipine dissolved in glacial acetic acid using p-naphtholbenzein as an indicator to a green end-point.

### **5.14 Crystal Structure (13)**

In the crystal lattice the almost flat dihydropyridine ring lies at a practically perpendicular angle to the nitrophenyl group, the ortho-nitro group facing away from the dihydropyridine ring.

### **5.15 Ultraviolet Spectrum (14)**

The ultraviolet spectra of nifedipine were taken with a Perkin-Elmer UV-spectrophotometer Lambda 5 at a concentration of 1.00 mg in 100 ml methanol, 0.1 N HCl and 0.1 N NaOH. The substance showed absorption maxima at 235 and around 340 nm in methanolic solution, and at 238 nm and around 340 nm in alkaline and acid solutions respectively.

The molecular extinction coefficients and  $A_{1\%}^{1\text{cm}}$  of nifedipine are reported to be

	<u>Methanol</u>	<u>0.1 N HCl</u>	<u>0.1 N NaOH</u>
Absorption Maximum	340 nm	338 nm	340 nm
	235 nm	238 nm	238 nm
$A_{1\%}^{1\text{cm}}$	145	165	165
	624	595	592
$\epsilon$	5010	5740	5740
	21590	20600	20510

The UV-spectra are shown in Fig. 3.

### 5.16 Infrared Spectrum

The infrared spectrum of nifedipine is given in Fig.4. The spectrum was obtained with a Perkin-Elmer 1420 Ratio Recording Infrared Spectrophotometer from a KBr pellet. The structural assignments may be correlated with the following band frequencies.



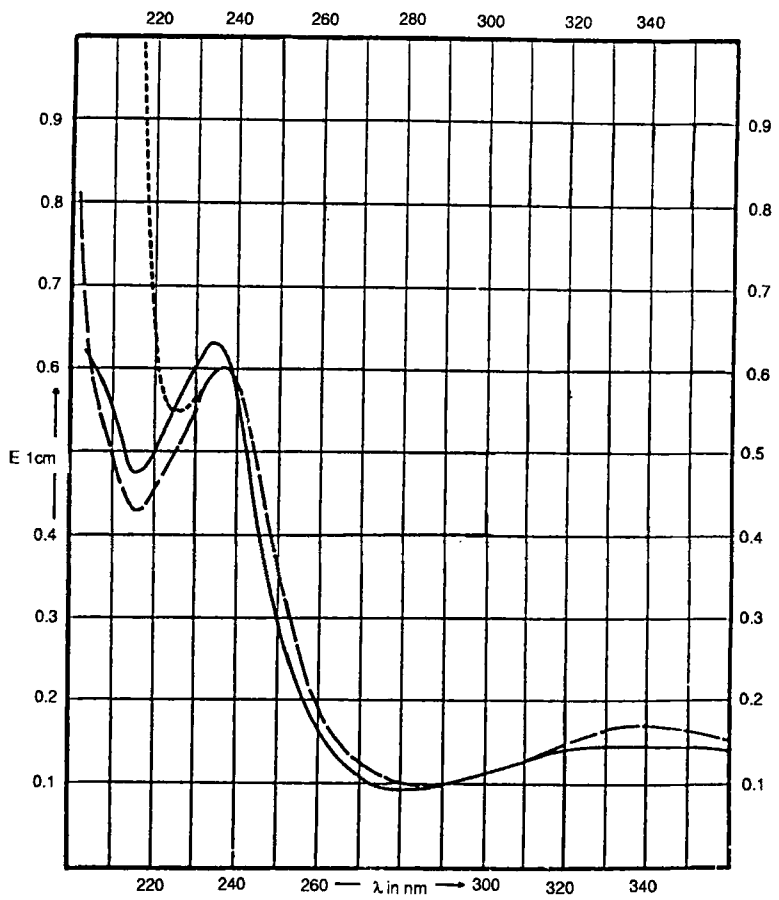


Fig. 3 (14)

— Methanol  
-- 0.1 N - HCl  
--- 0.1 N - NaOH

UV Spectra of Nifedipine in different Solvents

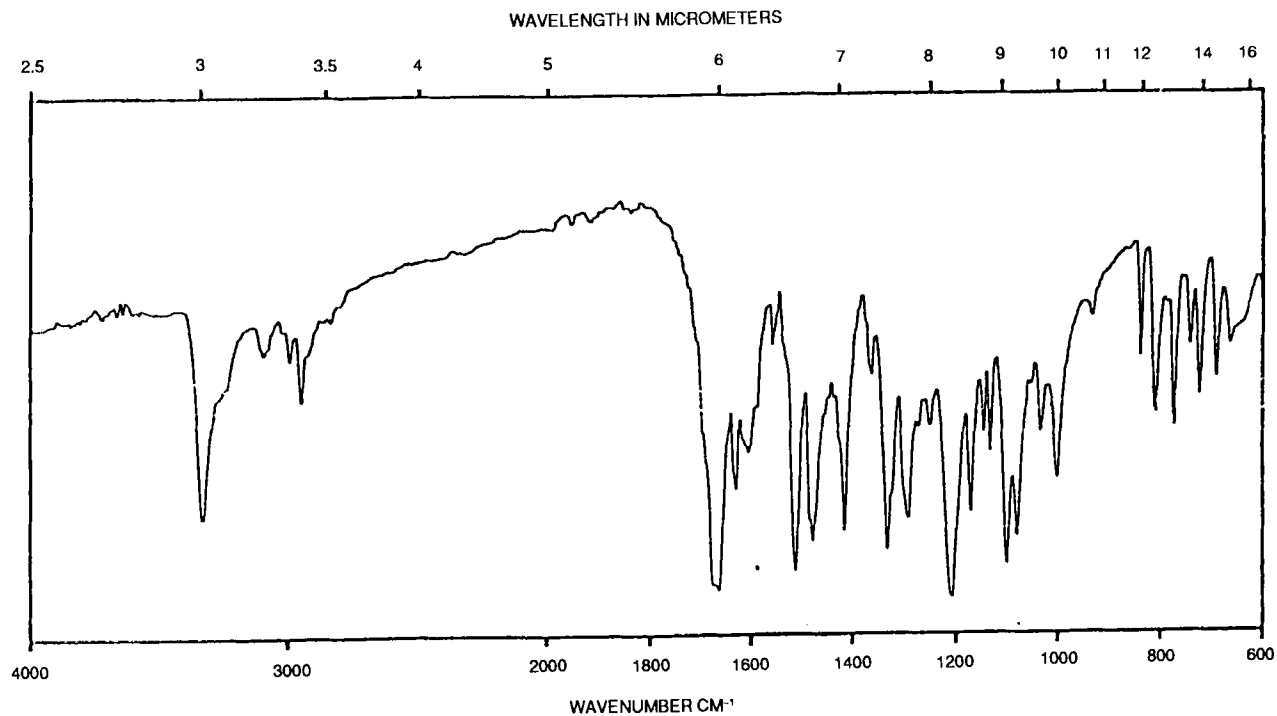
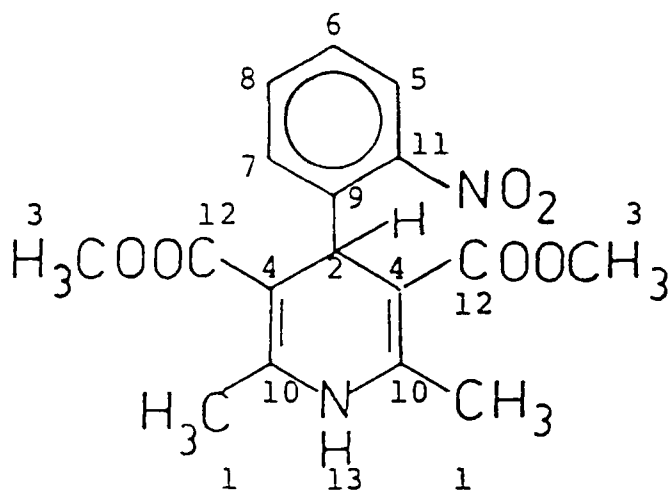


Fig.4  
IR Spectrum of Nifedipine, KBr Pellet  
Perkin-Elmer 1420 Spectrophotometer

<u>Frequency cm<sup>-1</sup></u>	<u>Assignments</u>
3331	NH stretching vibrations
3102	CH-aromatic
2931, 2842	CH-aliphatic
1689	C=O ester
1679	
1625	-C=C-aromatic
1574	
1530	NO <sub>2</sub>
1433	
1380	-C-CH <sub>3</sub>
1227	
1121	-C-O-ester

### 5.17 Nuclear Magnetic Resonance Spectrum

The nuclear magnetic resonance spectrum of nifedipine was obtained with a Bruker 250 MHz spectrometer, Model WM 250 in deuterated chloroform with a substance concentration of 200 mg/ml at 26°C using TMS as internal standard. The following spectral assignments of the structural formula of Fig. 5. are made for the spectrum reproduced in Fig. 6.

Nifedipine

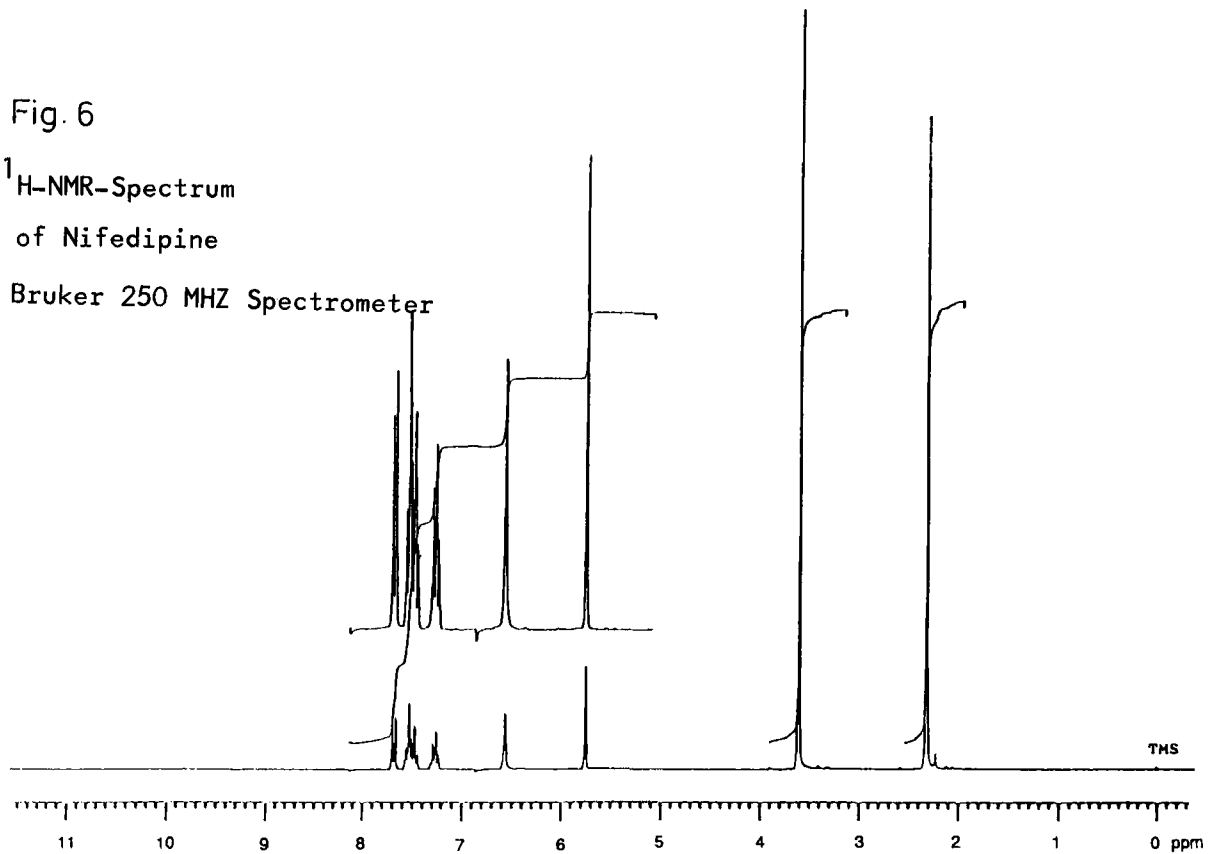
Structural Assignment of NMR - Signals

Fig. 5

Fig. 6

$^1\text{H}$ -NMR-Spectrum  
of Nifedipine

Bruker 250 MHz Spectrometer



<u>H-Atom in</u> <u>Fig.5</u>	<u>Chemical Shift</u> <u>ppm</u>	<u>Multiplicity</u>	<u>Number of H</u> <u>Atoms</u>
H-1	2.31	singulett	6
H-2	5.74	singulett	1
H-3	3.60	singulett	6
H-5	7.68	doublett	1
H-6	7.26	multipllett	1
H-7	7.50	doublett	1
H-8	7.51	multipllett	1
H-13	6.56	broad singulett	1

### 5.18 <sup>13</sup>C-NMR-Spectrum

The <sup>13</sup>C nuclear magnetic resonance spectrum was recorded with Bruker spectrometer WM 250. The substance was dissolved in deuterated chloroform (200 mg/ml) and spectrum obtained at about 26°C using deutrochloroform signals at 77 ppm as an internal standard. The following spectral assignments of the structural formula (Fig. 5) are made for the spectrum reproduced in Fig. 7. The decoupled spectrum is shown in Fig. 8.

Fig. 7

$^{13}\text{C}$ -NMR - Spectrum of Nifedipine  
Bruker Spectrometer WM 250

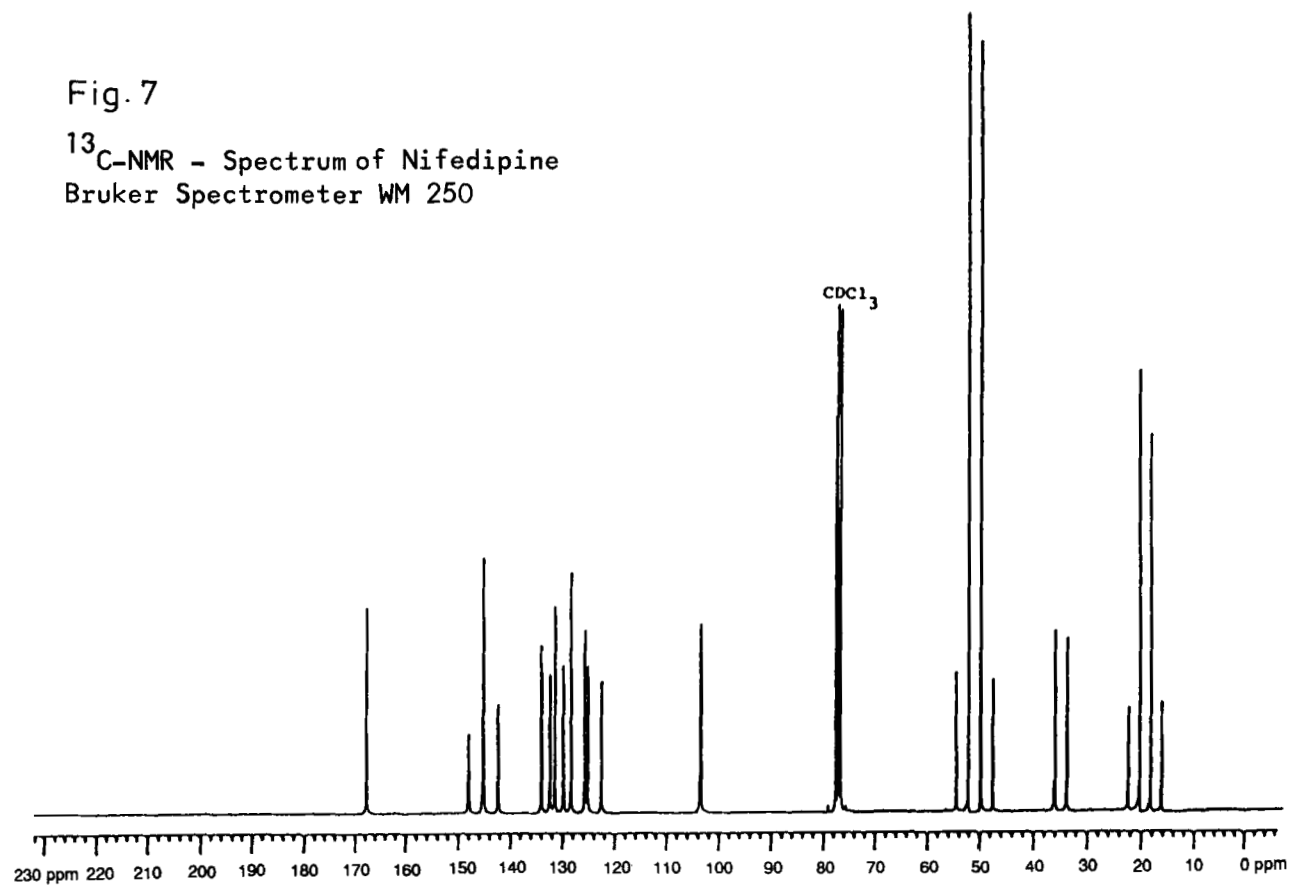
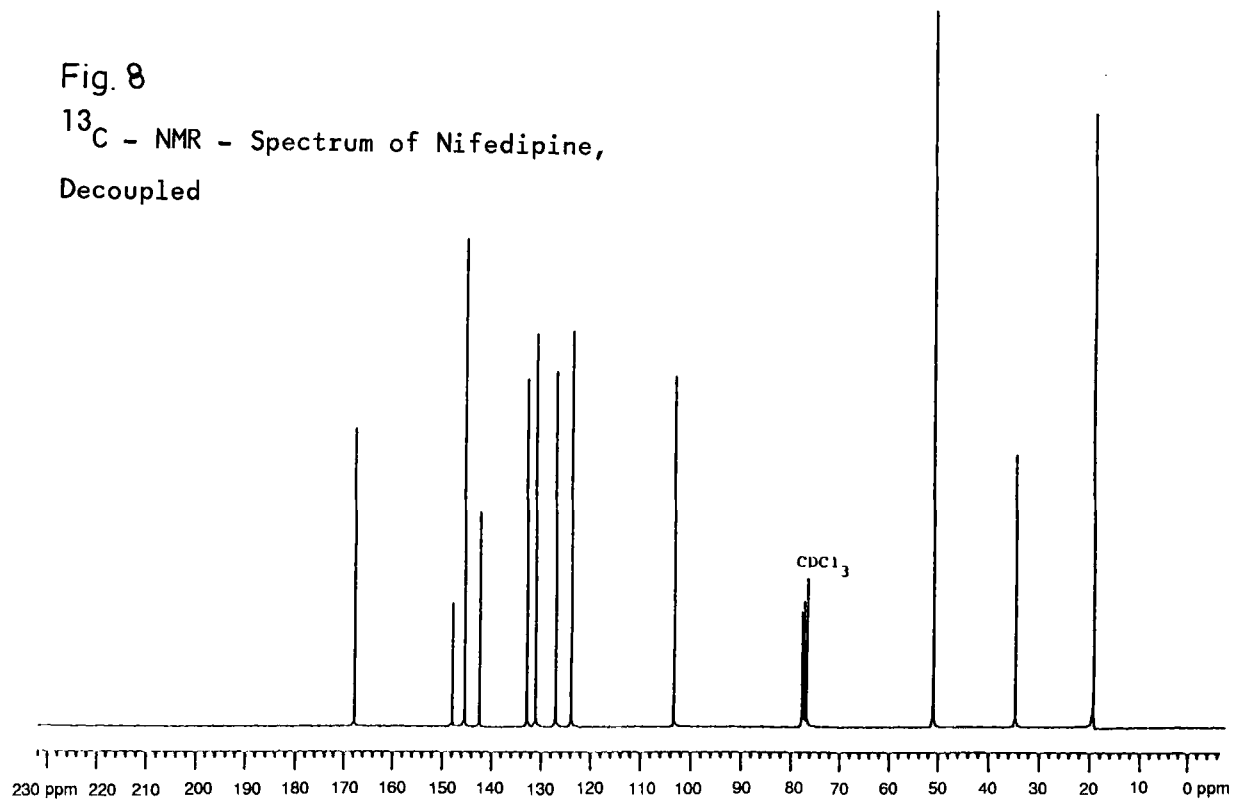


Fig. 8  
 $^{13}\text{C}$  - NMR - Spectrum of Nifedipine,  
Decoupled





<u>C-Atoms in</u> <u>Fig. 5</u>	<u>Chemical Shift</u> <u>ppm</u>	<u>Multiplicity</u>	<u>Number of C</u> <u>Atoms</u>
C-1	19.10	quadruplett	2
C-2	34.51	doublett	1
C-3	50.87	quadruplett	2
C-4	103.24	singulett	2
C-5	123.72	doublett	1
C-6	126.93	doublett	1
C-7	130.95	doublett	1
C-8	132.69	doublett	1
C-9	142.16	singulett	1
C-10	145.23	singulett	2
C-11	147.74	singulett	1
C-12	167.62	singulett	2

### 5.19 Mass Spectrum

The mass spectrum was recorded with a Varian Mat 311 mass-spectrometer using direct inlet in EI-mode at 70eV and source temperature of 200°C and direct inlet temperature of 115°C. The spectrum is illustrated in Fig. 9.

The ions of the spectrum can be correlated to the structure as following:

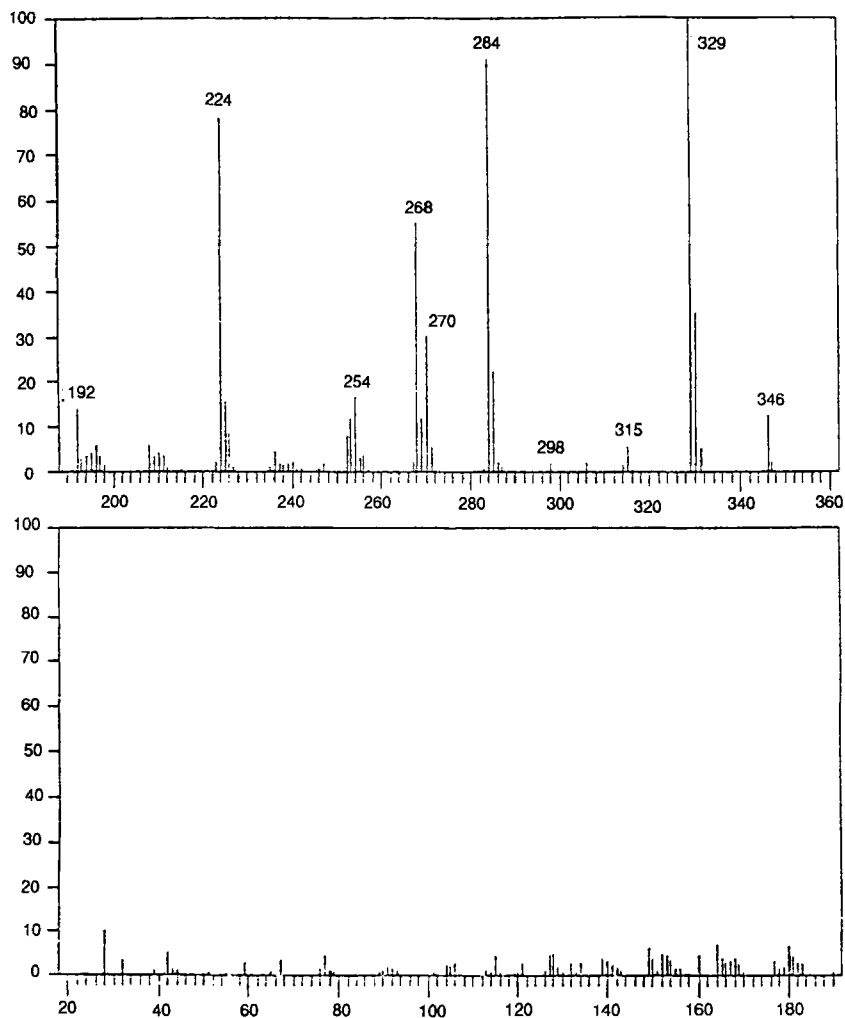


Fig.9  
Mass Spectrum of Nifedipine,  
Varian MAT 311 Mass Spectrometer

<u>Mass number</u>	<u>Structural assignment</u>
346	Molecular peak $M = C_{17}H_{18}N_2O_6$
329	$M-OH$
315	$M-OCH_3$
298	$M-OCH_3 \cdot OH$
284	$M-OCH_3 \cdot OCH_3$
270	$M-CO_2CH_3 \cdot OH$
268	$M-OCH_3 \cdot NO_2 \cdot H$
254	$M-OCH_3 \cdot OCH_3 \cdot NO$
224	$M-C_6H_4NO_2$
192	$M-C_6H_4NO_2 \cdot OH \cdot CH_3$

## 6. Colour and Identification Reactions (15)

The substances (50 mg) is dissolved in a solution of equal volumes of alcohol and 3 N hydrochloric acid (10 ml). About 0.5 g of zinc granules are added and the solution is allowed to stand for 5 minutes with occasional swirling. After filtering the solution 5 ml of 1 % sodium nitrite solution is added and the mixture is allowed to stand for 2 minutes. Subsequently 2 ml of 5 % ammonium sulfamate are added, shaken vigorously and finally 2 ml of a 0.5 % solution of N-(1-naphthyl)ethylenediamine dihydrochloride solution is added. An intense red colour develops which persists for not less than 5 minutes.

The nitro group of nifedipine could be reduced to hydroxylamine which forms with benzoyl chloride hydroxamic acid derivative. The derivative reacts with iron (III) chloride solution to give a deep red coloured solution.

About 25 mg substance are dissolved in 1 ml ethanol 90 %, 5 ml 1 % calcium chloride solution and 100 mg zinc powder are added, the suspension is stirred vigorously and heated for 10 minutes at 80°C in water bath. Afterwards the suspension is filtered and 3 ml filtrate are treated with 5 drops benzoyl chloride solution, shaken well for 1 minute and subsequently 10 drops of a 10.5 % ferric chloride are added under thorough mixing. A red coloured solution is obtained which turns yellow under addition of 2 N HCl.

A yellow solution with an absorption maximum at 330 nm is obtained when nifedipine (50 mg) is dissolved in dimethyl sulfoxide (1 ml). The yellow colour turns into tomato red colour with an absorption maximum at 451 nm when the solution is made alkaline with 2 N sodium hydroxide (5 drops). When an acidified dimethyl sulfoxide solution of nifedipine (30 mg nifedipine in a mixture of 2 ml glacial acetic acid, 2 ml dimethyl sulfoxide and 5 drops 2 N HCl) is treated with 20 drops of 2 % chromic (VI) oxide solution (200 mg CrO<sub>3</sub> in 10 ml glacial acetic acid + 10 drops 2 N HCl), a greenish-yellow colour is obtained (15).

## 7. Stability and Degradation

Nifedipine is a relative sensitive compound. Exposure to light, high temperature and presence of oxidizing agents yield predominantly two degradation products. This is illustrated in Fig. 10 (16a). Under the influence of visible and ultra-violet light nifedipine in solutions is converted into nitroso compound. Nifedipine on tlc pla-

In vitro degradation

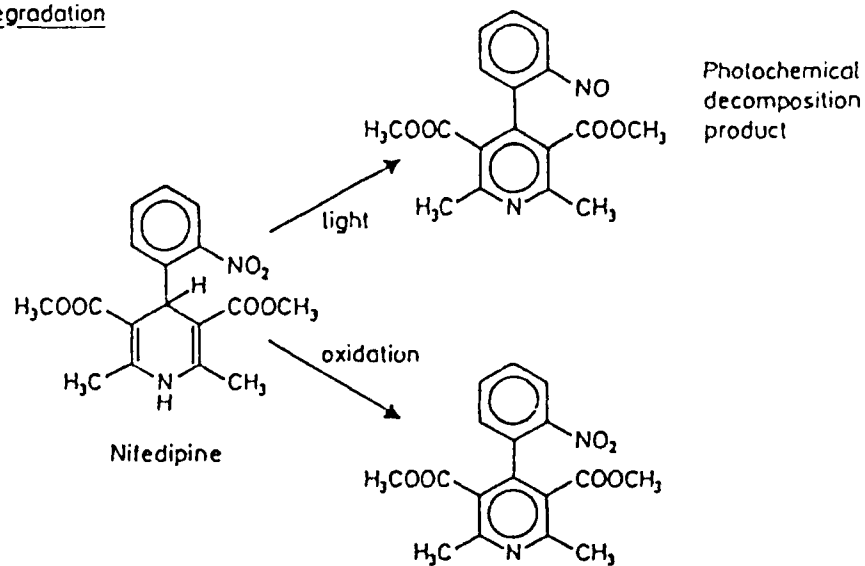


Fig. 10 (16a)

In Vitro Degradation of Nifedipine

tes is partially converted into the nitroso product within five to thirty minutes when exposed to day light and tube-light respectively. After 5 hours no unchanged nifedipine could be detected. Similar results were obtained when tlc plates are irradiated with UV light of 254 nm and 366 nm (16). In a second experiment nifedipine powder on a watch-glass was irradiated with a halogen lamp of 1000 W between 5 to 720 minutes. The conversion of nifedipine into the nitroso compound begins after six hours and is completed after months. Even after irradiation for a period of 11 months no nitro product could be detected. In weak hydrochloric acid solution nifedipine is already quantitatively converted into the nitroso derivative within one hour when exposed to the lamp-light of 300 W from a distance of 50 cm. In diffuse day light this process goes on gradually for hours (16). Nifedipine solutions are extremely photosensitive. On exposure to daylight its UV-VIS spectrum changes quickly. Under constant conditions nifedipine degrades by apparent first order reaction of non-radical type with a quantum efficiency of  $Q = 0.42$ . Nifedipine is affected by light of wavelengths below 450 nm. The photostability of a similar compound without the nitrophenyl group is remarkably good compared to nifedipine (17). Yellow coloured dilute alcoholic solutions of nifedipine (0.01 %) turn colourless when exposed to daylight, whereby a new absorption maximum of nitroso compound is formed at 280 nm. In table 1 the decomposition parameters of photodegradation of nifedipine are given under the influence of different light sources (17).

Table 1: Decomposition parameters K, t<sub>50</sub> and t<sub>90</sub> of nifedipine in alcoholic solution; photodegradation with different light sources

Light source	k (min <sup>-1</sup> )	t <sub>50</sub> (min)	t <sub>90</sub> (min)
spectrotest (xenon lamp) 700-372 nm	0.198	3.5	0.5
spectrotest (xenon lamp) 700-417 nm	0.040	17	2.5
day light, november	0.015	45	7
light bulb, 40 W	0.005	135	20

Figure 11 (17) illustrates this phenomenon further.

The decomposition velocity of nifedipine in solutions under the influence of light is remarkably high. This makes it impossible to work with the nifedipine solutions in daylight on account of considerable decomposition. Therefore all experimental work with nifedipine solutions should be performed under red light. Yellow light of sodium lamp is also suitable, as yellow or red light are not absorbed by nifedipine and consequently has no influence on its decomposition. The decomposition of nifedipine is a photolytic process. Oxygen and potassium iodide have no influence on the degree or velocity of decomposition process (17). It is reported that the nitrophenyl

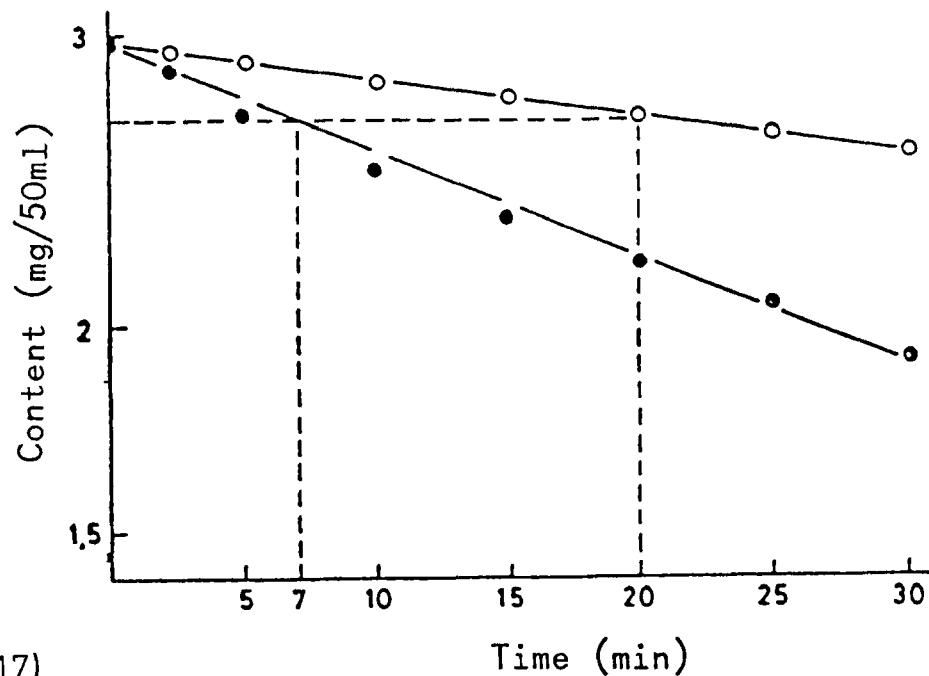


Fig. 11 (17)

Decomposition of Nifedipine, dissolved in absolute ethanol, in Relation to the Light Source

- diffuse daylight November
- light bulb 40 Watt, 11 cm distance



rest in nifedipine molecule is responsible for its quick decomposition (17).

The photostability of nifedipine is influenced by several parameters such as wavelength and intensity of light exposure, concentration of solutions, solvent effects and quality of vials. Temperature, pH and ionic strength have no influence on stability. Depending on the light sources, for example tungsten or xenon lamp, the reaction rate constant differs by the factor 40 while the influence of the initial concentration on reaction rate is characterised by the factor 8. Solvent mixtures, ethanol and 1,2-propanediol-water mixtures decrease the degradation. Normal brown glass vials could not hinder the light induced instability of nifedipine. 10 % nifedipine already degraded within 20 minutes. Nifedipine crystals are more stable than its solutions, because the light effect is a surface phenomenon. After exposure of nifedipine to the light of xenon and tungsten lamps the nitroso compound is formed as decomposition product which could be verified through tlc and infrared spectroscopic investigations. The NH-valence bond vibration band of dihydropyridine ring disappears after irradiation of nifedipine with light sources (18). Nifedipine remains in a solvent mixture of ethanol-water, 1:1 stable when protected from light. The solution of nifedipine in polyethylene glycol 600 remains stable for 5 months. The temperature has no influence on the nifedipine stability under protection from light. Nifedipine solutions kept at 50°C for five months remained stable under complete exclusion of light (18). Concentration-dependence of the rate of decomposition of nifedipine has been esta-

blished. A solution containing 120 mg nifedipine in 100 ml showed 50 % decomposition after 2 days of light exposure, whereas a solution of 6 mg nifedipine per 100 ml decomposed to 50 % already after 1 hour (18).

The parameters pH between 2 and 12 and ionic strength have no influence on the stability of nifedipine.

The influence of various excipients, such as PVP, PEG(20)-sorbitanmonolaurate, 1,2-propandiol, ethanol and PEG 200 has been investigated. Solvent mixtures with a dielectric constant greater than 72 have a positive effect on the stability of nifedipine, whereas those with a dielectric constant between 22 and 28 decrease its stability. The stability of nifedipine has been markedly increased using PVP as complexing agent. Nifedipine solutions in brown-glass ampulles are decomposed to 10 % of its value already after 20 minutes, whereas in white glass ampulles 50 % of nifedipine is decomposed after 20 minutes (18). The photolytic decomposition of nifedipine in crystalline form is not substantial, reaching only a value of 20 %. This is accounted for the inability of a deeper penetration of light rays in the crystalline surface.

The decomposition product Dimethyl-4-(2-nitrosophenyl)-2,6-dimethyl-pyridine-3,5-dicarboxylate is formed in normal day light and limited upto 0.2 % in nifedipine by USP XXI. The other product Dimethyl 4-(2-nitrophenyl)-2,6-dimethylpyridine-3,5-dicarboxylate is considered to be synthetic impurity in nifedipine, may also be formed in ultraviolet light and could be present upto 0.2 % in nifedipine according to USP XXI (11).

The stability of nifedipine in cardioplegic solutions was studied under different storage conditions. Nifedipine degraded more rapidly at 25°C than at 4°C. Its concentration declined to less than 90 % of original potency by about 6 hours after preparation. There was no significant degradation during the simulated infusion regardless of light exposure or flow rate. It was thus concluded that cardioplegic solutions containing nifedipine should be prepared immediately before the surgical procedure, refrigerated until use and protected from light until administration (19).

The photosensitivity of nifedipine drops was studied. Results obtained using HPLC showed that nifedipine solutions prepared by diluting the drops and maintained at normal conditions during therapeutic use are stable for 10 minutes resulting in a degradation of less than 5 % during exposure at ambient illumination (20).

The physicochemical stability of coprecipitate systems of nifedipine with urea, polyethylene glycols and povidones was studied. Chemical instability of nifedipine was found when coprecipitated with urea. High humidity affected the dissolution and availability of nifedipine with povidones. Polyethylene glycol coprecipitates seemed to be stable under all conditions (21).

Daylight decomposition of nifedipine to the nitrosopyridine derivative and to the nitropyridine derivative under UV irradiation has been reported (22).

The breakdown-rate of nifedipine in laboratory light for four hours was examined in distilled water, plasma and whole blood. The light protective effect of whole blood is very marked, allowing only an 11 % decrease in nifedipine level due to photodegradation over four hours compared to a 62 % decrease in plasma and 79 % decrease in aqueous solution. Controls of whole blood or plasma wrapped in foil show no reduction in nifedipine throughout the 4 h. For 25, 50 and 100 ng/ml nifedipine in whole blood the breakdown is nonexponential and non-linear, however, in plasma and distilled water break-down of nifedipine is exponential (23).

## 8. Methods of Analysis

### 8.1 Titrimetry

100 to 150 mg of nifedipine are weighed accurately and dissolved in 20 ml glacial acetic acid, then 10 ml dilute sulfuric acid are added and the mixture is heated in water-bath at 70°C. The mixture is titrated against 0.1 N ammonium cer (IV)-sulfate solution after addition of 0.1 ml ferroin indicator from red to blue end-point. 1 ml 0.1 N ammonium cer (IV)-sulfate solution corresponds to 17.31 mg nifedipine (15).

## 8.2 Polarography

Direct current and alternating current polarographic methods were developed to determine the content uniformity and photostability of nifedipine in pharmaceuticals. Form and position of the polarographic curves are pH-dependent. At pH 1.65 and a voltage of -0.75 V versus Ag/AgCl electrode a suitable diffusion current is obtained. Nifedipine receives 4 electrons per molecule during cathodic reduction. A mixture of 0.1 N HCl + ethanol, 1:1 was used as supporting electrolyte. With alternating current polarography nifedipine peak appears at -0.710 V which disappears in course of photolytic decomposition and a new peak appears at -1.22 V. This allows a specific determination of nifedipine and its decomposition product. The standard deviation of the method is 0.28 % in the concentration range of  $10^{-4}$ M. The detection limit is given as  $5 \times 10^{-7}$ M (24).

## 8.3 Visible and UV Spectrophotometry

Nifedipine is quantitatively determined in methanolic solution through the measurement of its absorbance at 350 nm (11). Nifedipine is also determined in course of the examination of uniformity of dosage units in methanolic solution through spectrophotometry at 350 nm. The assay of nifedipine in capsules is performed after dissolving its contents in methylene chloride, tlc separation, scratching off the zones, elution of the drug with methanol and its subsequent determination at 340 nm (11).

Nifedipine shows a sharp maximum at 235 nm and a broad band between 325 and 370 nm. The nitroso pyridine compound of nifedipine has maxima at 280 and 310 nm in ethanol and at 279 and 315 nm in alkaline (0.1 N NaOH) and acidic (0.1 N HCl) solutions. The nitropyridine derivative shows maxima at 277 nm in acidic and at 273 nm in alkaline solutions (16).

#### 8.4 Fluorimetry

A fluorimetric assay of the coronary active compound nifedipine in plasma is described which involved the reduction of the nitro group to an amino group by  $\text{TiCl}_3$  and the oxidation of the dihydropyridine ring to the pyridine ring by exposure at 360 nm. This amino derivative is coupled with o-phthaldialdehyde which leads to a product with increased fluorescence intensity. The fluorimetric determination is carried on at a excitation wavelength of 390 nm and the emission wavelength of 520 nm with a Zeiss spectrofluorometer using xenon lamp. There is a linear relationship between fluorescence intensity and nifedipine concentration in the range of 1-10  $\mu\text{g/ml}$ . The detection limit is given with 0.3  $\mu\text{g/ml}$  plasma, whereas 3 ml plasma should be used for measurements (25). The main metabolite of nifedipine (4-(2-nitrophenyl)-2-hydroxy-methyl-5-methoxycarbonyl-6-methyl-pyridine-3-carboxylic acid) can be also determined flurometrically in the same way by fractional extraction of plasma samples. At first nifedipine is extracted at pH 7.5 and then metabolite at pH 2.0. Since only the main metabolite and nearly no

Nifedipine is excreted via urine, only the main metabolite is determined through fluorimetry (25,26).

## **8.5 Chromatographic Methods**

### **8.5.1 Thin Layer Chromatography**

Nifedipine is separated on 0.5 mm thick silica gel plates with a mobile phase of ethyl acetate and cyclohexane, 1:1 at about  $R_f$  0.3. The related compounds of nifedipine are also separated on silica gel tlc plates with diisopropyl ether as the solvent (11). Ebel and coworkers tested 17 different solvent systems and found a mixture of chloroform-cyclohexane, 95:5 and silica gel 60 tlc plates optimum for the separation of nifedipine and its derivatives (16). After separation the tlc plate is sprayed with  $TiCl_3$  solution for the reduction of the nitro groups. The plate is dried with cold air and further cooled at  $-20^\circ C$ . Subsequently the plate is reacted in a chamber with the sodium nitrite solution for diazotisation of the amino groups and again dried with stream of cold air. Finally the plate is sprayed with Bratton-Marschall coupling reagent. Nifedipine shows a reddish brown spot at  $R_f$  0.12, nitro and nitroso-derivatives appear as yellow spots at  $R_f$ -values 0.16 and 0.27 respectively (16). Nifedipine and its decomposition products obtained after exposure to various light sources were separated on silica gel 60 F - 254 Merck tlc plates with a mobile phase of chloroform + diethyl amine + cyclohexane, 60:15:75. The tlc plates were developed until the solvent front reached a length of

10 cm and then the spots were detected at 254 nm and 366 nm (18). The substance appears at R<sub>f</sub>-value of 0.18 as yellow spot in UV 254 nm light. The drug is separated from the contents of Adalat capsules with the above mentioned chromatographic system. Nifedipine and other excipients are detected by spraying the plate with an alkaline potassium permanganate solution (15). The metabolites of nifedipine in urine samples were extracted with chloroform and separated on silica gel plates with the solvent system toluene-glacial acetic acid-water, 5:5:1 (27).

#### 8.5.2 Gas Liquid Chromatography

Nifedipine and its two derivatives nitroso- and nitro compounds were analysed through GLC using a 1.5 m glass column 3 % OV-17 on chromosorb G, AW-DMCS, 80-100 mesh, oven temperature 250°, nitrogen as carrier gas and FID. The net retention times for nifedipine, nitroso- and nitro compounds are given as 790, 158 and 250 seconds respectively. The analysis on a 3 % SE 30 on chromosorb G, AW-DMCS (80/100 mesh) column resulted in retention times 143 S for nifedipine, 48 S for nitroso compound and 68 S for nitro compound. The compounds did not show any thermal instability during the gas chromatography (16). 1.8 m glass column containing 5 % SE 30 on chromosorb, AW/DMCS, 80-100 mesh, oven temperature 280°C isotherm, FID and nitrogen as carrier gas have been used for the analysis of nifedipine where nifedipine eluted at a retention time of 183 seconds (15). The GC method with FID allows the analysis of nifedipine in the  $\mu\text{g}$  concentration range (15).



Gas chromatographic determination of nifedipine and one of its metabolites using electron capture detector has been reported (28). The GC glass column (180 cm x 2 mm ID) was filled with 2 % OV-17 on gaschrom Q, 80-100 mesh, and conditioned for 24 h at 275°C with a nitrogen flow-rate of 25 ml/min. <sup>63</sup>Ni ECD was used and the operating conditions were: column temperature 240°, injector temperature 240° and detector temperature 290°C. Under these conditions the metabolite (obtained through oxidation of nifedipine with potassium permanganate at pH 9), diazepam internal standard and nifedipine had retention times of 1.3, 2.4 and 4.0 minutes respectively (28). The calibration curves for nifedipine and the oxidation product were linear between 0 and 100 ng/ml with regression coefficients of 0.9983. The minimum detectable concentration was found to be about 1 ng/ml for both nifedipine and the metabolite. The within-run coefficient of variation was 2.1 % for 50 ng/ml and 2.8% for 100 ng/ml nifedipine. Combined GC-mass spectrometry confirmed that nifedipine is stable on the GC column (18). GC with FID has been used by Testa et al. (22) for the analysis of nifedipine.

In course of pharmacokinetic studies of nifedipine a gas-chromatographic method with electron capture detection has been applied. A DB 1.5 m column operated at 160°C column temperature, 250°C injector temperature and 300°C detector temperature was used with helium as carrier gas. The oven temperature was programmed from 160 to 270°C with increment of 10°C/min (29,26). For the determination of nifedipine in biological fluids several gas chromatographic methods have been described, with either electron-capture

detection or selective ion monitoring through mass spectrometry. Kleinbloessem (30) and coworkers report that the thermostability of nifedipine under the gas chromatographic conditions applied (230-250°C) represents a serious problem, since nitroderivative of nifedipine is formed in non-reproducible amounts. Therefore Kondo et al. (31) and Higuchi and Shiobara (32) oxidised nifedipine prior to GC analysis, despite the loss of selectivity. The nifedipine content of plasma samples was analysed through capillary gas chromatography with EC detector (33). In another GLC method with ECD upto 2 ng/ml nifedipine could be detected in plasma (34). Nifedipine and its nitropyridine derivative in human plasma were analysed through GLC. A 63 Nickel electron-capture detector and a glass 1.83 m x 2.0 mm ID column packed with 2 % OV 17 on gas-chrom Q (100-120 mesh) and argon-methane (95:5) carrier gas with a flow-rate 32 ml/min were used. Injection port temperature was maintained at 245°C, column temperature at 235°C and detector temperature at 280°C (35). Due to the thermoinstability of nifedipine at higher temperatures a controversy exists in the literature concerning the detection and quantification of this substance in complex matrices. The authors maintain that the possibility of thermal degradation of nifedipine to its nitroderivative during the chromatographic process cannot be ruled out with GLC at higher temperatures (230-250°C). This procedure is applicable to the simultaneous determination of nifedipine, nitropyridine and nitrosopyridine derivatives. Linearity of the procedure was established for 10-200 ng nifedipine/ml plasma and 10-50 ng nitropyridine derivative/ml plasma. The detection limit is given as 1 ng/ml. The relative

standard deviation for nifedipine determination lies between 1.1-4.5 % (200-10 ng/ml) and for nitropyridine derivative between 1.9-11.9 % (50-5 ng/ml) (35).

Nitropyridine derivative found in plasma samples is the product of metabolism and does not arise during the preparation, extraction etc. of plasma samples (35).

Determination of nifedipine in human plasma by capillary gas chromatography with a nitrogen detector is reported (36). GC was performed on a 13 m x 0.31 mm ID cross-linked methylsilicone fused-silica column, film thickness 0.52  $\mu$ m with helium carrier gas flow of 6 ml/min. A nitrogen-phosphorous ionization detector, a Hewlett-Packard capillary on-column injection port system 19320 H was used. Operation temperatures were: injection, 230°; oven, 210°C; detector, 310°C. Detector conditions were set on a high response: helium make-up gas, 20 ml/min; hydrogen, 3.5 ml/min; air, 75 ml/min. Nifedipine was extracted from plasma at basic pH with toluene and nitrendipine was used as an internal standard. The minimal detectable concentration was 0.5 ng/ml plasma. The standard curve was linear in the range of 2-300 ng/ml nifedipine. The within-analysis coefficient of variation was 3.9-10.4 % and day-to-day coefficient of variation was 3.8 %. Under the chromatographic conditions used nifedipine, the nitropyridine metabolite of nifedipine and nitrendipine internal standard gave fully resolved, essentially symmetrical peaks eluted in 10 min (36).

A study of nifedipine photodecomposition in plasma and whole blood using capillary GLC with electron capture detection has been performed (23). The column fitted was a fused silica open tubular SE 30 column, 12 m x 0.33 mm ID. The carrier gas helium flowed at 2.5 ml/min and nitrogen make up gas at 30 ml/min. The injection mode was splitless and the temperature settings used were: detector 300°C, injector 250°C, temperature programme: 100°C to 200°C at 40°C/min, 200° to 260°C at 10°C/min and hold for 1 min. The calibration curve for nifedipine was linear from 0-150° ng/ml and the detection limit was about 0.5 ng/ml. There was no interference from nitropyridine derivative or other metabolites of nifedipine (36). Lesko and coworkers have described a rapid GC method with electron capture detection for quantitation of nifedipine in serum (37). Plasma concentrations of nifedipine have been determined through gas chromatographic methods in course of bioavailability studies (38,39,40).

### 8.5.3 High Performance Liquid Chromatography

Nifedipine has been separated on a Bondapack C18-NH<sub>2</sub> column, 30 cm x 3.9 mm using chloroform as mobile phase at a flow rate of 1.5 ml/min and detected at 254 nm. Nitroso-pyridine derivative and nifedipine eluted at retention times of 4.30 and 7.30 minutes respectively (15). Nifedipine and the related substances nitroso- and nitropyridine derivatives in nifedipine can be analysed through HPLC on a reversed phase Nucleosil C18, 5 µm column with a mobile phase acetonitrile + methanol + water, 9:36:55. The flow-

rate was maintained at 1.0 ml/min, column temperature at 40°C and the detection wavelength was set at 270 nm. The sample and reference substances were dissolved in methanol and diluted with the same solvent and 20  $\mu$ l were injected into liquid chromatograph. The relative retention times are given for nifedipine: 1.0, nitropyridine derivative: 0.72 and nitrosopyridine derivative: 0.86 (41).

Nifedipine and its metabolic product nitropyridine derivative were analysed in human plasma samples with HPLC. The HPLC system consisted of a Nucleosil C 18 column, particle size 5  $\mu$ m, 200 x 6 mm ID, mobile phase water-methanol-acetonitrile, 55:36:9, flow-rate 1 ml/min and a UV detector operated at 235 nm. After extraction of the plasma samples with toluene, the solvent was evaporated to dryness and the residue reconstituted in methanol. 100  $\mu$ l were injected into liquid chromatograph (35).

A HPLC method was developed for the assay of nifedipine in plasma and its main metabolite, the nitropyridine derivative with one ester moiety of the side chain hydrolysed in urine. After liquid-liquid extraction nifedipine was chromatographed on a MOS-Hypersil. Column (Shandon, UK) 100 m x 2.8 mm ID, 5  $\mu$ m particle size and detected at 238 nm. The mobile phase consisted of 0.05 M acetate buffer, pH 4.0 - acetonitrile, 7:5 and the flow-rate was 1.0 ml/min. The metabolite in urine was separated on a Rad-pak C8 cartridge, 100 mm x 5 mm ID, particle size 10  $\mu$ m and detected at 290 nm. The mobile phase had the composition acetonitrile-water, 1:3 + 0.009 M cetrimide and the flow-rate was set at 4.0 ml/min. The method was sensi-

tive to 2 ng nifedipine per ml plasma and the calibration curve was linear to at least 400 ng/ml. Relative standard deviation did not exceed 8.5 %. There was no interference from other photodecomposition products or metabolites. The method for the analysis of metabolites was sensitive to 0.02  $\mu$ g/ml urine, standard curve was linear upto 5  $\mu$ g/ml and the relative standard deviation did not exceed 5 % (30).

Another HPLC method for the determination of nifedipine in plasma is described. A Zorbax ODS, 4-6  $\mu$ m, 250 x 4.6 mm ID reversed phase column was used at 55°C. The mobile phase consisted of 0.01 M disodium hydrogen phosphate buffer-methanol, 45:55. Before mixing the pH of the buffer was adjusted to 6.1 with 50 % phosphoric acid. The flow-rate was 0.8 ml/min and the wavelength was set at 280 nm. The plasma samples were extracted with a mixture of chloroform-acetone 1:1, solvent evaporated to dryness, the residue reconstituted in the mobile phase containing butamben as internal standard and 20 to 30  $\mu$ l of the solution were injected into HPLC. Nifedipine and internal standard were well separated from endogenous substances. The relative recovery of nifedipine from plasma containing 40 ng/ml was estimated by comparing it with the recovery from an aqueous sample and was found to be  $99.1 \pm 3.2$  %. The detection limit is reported to be 5 ng/ml plasma (42).

HPLC has also been applied in monitoring the in vitro dissolution rate of nifedipine formulations. A Hypersil OD 5,5  $\mu$ m, 250 x 4.6 mm column at 35°C was used at a flow-rate of 1.0 ml/min. The mobile phase methanol - 0.01 M disodium hydrogen phosphate, adjusted to pH 6.5 with phosphoric acid, 55:45 was used and the detection wave-

length was set at 320 nm. The calibration curve was linear between 180-1800 ng/ml (43).

The assay of nifedipine in human plasma using an automatic HPLC procedure through a column switching technique with only one step dilution is described. Because of its sensitivity of 2-3 ng/ml and its good reproducibility the method is suitable for in vivo drug level monitoring and pharmacokinetic studies (44). A hyperchrome column, 125 x 4.6 mm, filled with Shandon Hypersil ODS 5,5  $\mu$ m connected with a precolumn, 40 x 4.6 mm filled with perisorb RP 18 was used. The mobile phase consisted of a 0.01 M disodium hydrogen phosphate buffer adjusted to pH 6.1 with phosphoric acid and methanol, 53:50; the flow-rate was 1.0 ml/min and the UV detection was set at 236 nm. The flow-rate of the wash-liquid (0.01 M disodium-hydrogen phosphate, pH 6.1) in the column-switching technique was kept at 2.0 ml/min (44).

HPLC of nifedipine, its metabolites and photochemical degradation products was performed on a 300 mm x 4.0 mm in U Bondapak C18 column, 10  $\mu$ m, at room temperature. A precolumn of Bondapak C18 corasil was used for the analysis of animal plasma. The flow-rate was 2 ml/min and UV detection was done at 254 nm. The mobile phase consisted of 0.01 M disodium hydrogen phosphate buffer, adjusted to pH 6.1 with 50 % phosphoric acid and methanol, 45:55. Nifedipine, its metabolites in plasma and its photochemical degradation products were well separated. This mobile phase allowed the separation of drugs from endogenous plasma substances or from the components of the pharmaceutical preparations as well as the use of 4-dimethyl-aminoben-

zaldehyde as a suitable internal standard. The minimum detectability of all compounds with the described procedure was 10 ng. Precision over a 30-day period using different plasma samples was  $\pm 4,1 \%$  (45).

Determination of nifedipine in human plasma by HPLC with electrochemical detection is reported. Chromatography was performed on a Unisil pack C18 column, 5  $\mu\text{m}$ , 150 x 4.6 mm I.D. using the mobile phase methanol-tetrahydrofuran-0.05 M phosphate buffer (pH 3.0), 660:10:330 at a flow-rate of 0.8 ml/min and at 20°C. An electrochemical detector model VMD 501 (Kyoto, Japan) was set at the potential 0.95 V versus Ag/AgCl reference electrode. The detection limit for a standard solution injected at 0.95 V was approximately 40 pg. A toluene extract of an alkalinised plasma sample was chromatographed using diethyl 1,4-dihydro-2,6-dimethyl-4-(2-nitrophenyl)pyridine-3,5-dicarboxylate as an internal standard. The recovery of nifedipine from plasma was about 100 % and detection limit in plasma was 2 ng/ml using 0.5 ml of sample. The assay gave linear response over the concentration range 5-400 ng/ml in plasma. Photodegradation products and metabolites of nifedipine did not interfere in the analysis (46).

#### 8.5.4 Gas Chromatography - Mass Spectrometry

Combined GC-mass spectrometry has been applied to examine the stability of nifedipine on GC column in course of determination of nifedipine in blood plasma. A Joel B 100 mass spectrometer connected to a gas chromatograph with ionization voltage 25eV and ionization current 300  $\mu\text{A}$  was used. The GC column was a 1 m 3 % OV 17 on gas-chrom Q



operated at 220°C. GC-MS confirmed that nifedipine is stable on the GC column. The GC-MS of the light-degradation product showed a molecular ion 18 mass units smaller than that of nifedipine and consistent with the formula of nitrosopyridine derivative. The GC-MS of the oxidation product was in agreement with the structural formula of nitropyridin derivative (28).

A GC-MS method for the bioavailability study of different sustained-release nifedipine formulation has been reported. A 2 m glass column filled with 1 % SE 30 on gaschrom Q 180-100 mesh, was operated with a temperature programme from 240 to 290°C with 60°C/min and helium carrier gas flowed at 45 ml/min. The mass spectrometric detection was done through single ion monitoring m/e 330 for nifedipine and m/e 284 for internal standard diazepam, using a MAT 44 mass spectrometer, 80 eV and 0.8 mA. Nifedipine was extracted from plasma with toluene, the solvent evaporated and the residue was reconstituted in acetone. The retention times for nifedipine and diazepam internal standard were found to be 1.1 and 1.3 min respectively. The calibration curve was linear between 5-500 ng per ml plasma, detection limit was 5 ng/ml, reproducibility of spiked plasma 100 % and the relative standard deviation lying between 5.3 - 10.0 % (43).

Higuchi and Shiobara have also reported a gas chromatography - mass spectrometric method of determination of nifedipine in plasma samples (32).

### **9. Radioactive Measurements of C-14 Labelled Nifedipine in Body Fluids**

Experimental studies in animals on pharmacokinetics and biotransformation of radioactive labelled nifedipine were carried on (47). In another study clinical investigations on the pharmacokinetics of radioactive labelled nifedipine in human test persons were performed (48). Following intravenous administration of C-14 labelled nifedipine in rats and dogs the C-14 radioactivity eliminated in urine and faeces was measured in a liquid szintillation counter. Solid samples were burnt in oxygen and residue taken in a 10 ml szintillator consisting of 850 ml toluene, 150 ml methanol + 8 g butyl PBD. For liquid samples a szintillation mixture of toluene + dioxane + ethanol, 1:1:1 to which 8 g butyl PBD had been added, was used. Human body fluids were also treated likewise and C-14 activity was measured with liquid szintillation counter (46).

A radioreceptor assay of nifedipine has also been reported (49).

### **10. In Vitro-Dissolution**

The specifications of the manufacturer Bayer, Germany demand that nifedipine sustained-release tablets should liberate in in vitro dissolution test after 2 hours between 40-70 % of the drug and after 6 hours at least 65 %. In case of tablets 70 % drug should have been dissolved after 30 minutes (50).

In vitro dissolution of nifedipine from 3 dosage forms was correlated with in vivo AUC following administration of 1 to 10 healthy subjects. It was suggested that in assessing the quality of nifedipine formulations at least 80 % of the drug be dissolved within 20 minutes in artificial gastric juice utilizing USP XX paddle method (51).

In vitro dissolution behaviour of two nifedipine sustained-release preparations was studied. A disstest CE 6 (Sotax, Basel, Switzerland) dissolution apparatus according to the flow-through method of Langenbucher was used. The dissolution medium for the first hour consisted of 0.08 N HCl, pH 1.2 and for the hours 2 to 8 it comprised of 0.05 M phosphate buffer solution pH 6.8. The flow-through rate was set at 1000 ml per hour. Nifedipine in both formulations liberated at a uniform rate and reached the value of 80 % after 8 hours (43).

An automatic system for the dissolution of slightly soluble drug substances is described. Aqueous dissolution medium is pumped through a flow-through cell of variable design. The drug substance in the cell is thus partially dissolved. In a separate extraction compartment the dissolved amount is extracted into the chloroform phase and the two phases are separated subsequently. The chloroform phase serves as a sink for the slightly soluble substance and will be used for the spectrophotometric determination of the dissolved substance. The system was successfully used for the dissolution of nifedipine. Because of the bad substance wettability flow-through cells with stirrer produced better results than those without stirrer. Nifedi-

pine microfein substance, nifedipine fast tablets 10 mg and nifedipine sustained-release tablets 20 mg were analysed for in vitro dissolution through this apparatus (52).

### **11. Drug Metabolism and Pharmacokinetics**

Nifedipine will be completely absorbed in gastro-intestinal tract by healthy test persons as well as patients. After sublingual administration resorption takes place already in mouth mucosa. The rate of resorption depends upon the type of formulation administered (48). After peroraler application of nifedipine capsules the drug is detectable in plasma after 15 minutes. In sublingual mode of administration nifedipine is present in plasma after 5-10 minutes. The maximum plasma concentrations are reached between 60-120 min (53,54). Fig. 12 shows typical concentration-time curves of nifedipine in human plasma after administration of 10 mg Adalat capsule and 10 mg Adalat tablet T 10 (54a). In case of sustained-release formulations the drug is already present after 15 to 30 minutes and maximum plasma concentrations are obtained between 2-4 hours (54) and therapeutic active levels of about 11 ng/ml are present still after 12 hours.

Foster and coworkers (55) have reported the results of unchanged nifedipine concentrations in plasma of 25 healthy test persons after administration of nifedipine capsules, sustained-release tablets and intravenous application. The persons received 1 mg intravenously or 10 mg by the intestinal route. Due to the low dosage ra-

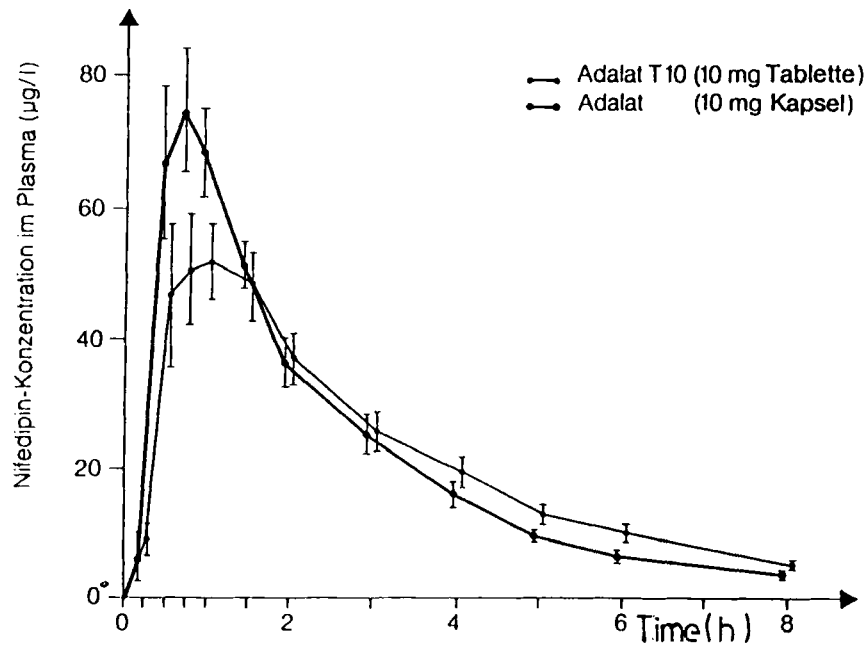


Fig. 12 (54a)

Nifedipine Concentration in Human Plasma (18 healthy volunteers) after Administration of Adalat 10 mg Capsule and Adalat 10 mg Tablet (T10)

radioactive methods were used for determining the unchanged substance and its metabolites in the serum, urine and faeces. Further the biotransformation was studied. Independent of the type of enteral administration a total of 90 % of the administered activity is absorbed. A comparison of the absorption rates following the administration of clinically usable administration forms (gelatine capsules, swallowed whole or chewed) showed the marked superiority of the sublingual administration of the gelatine capsules. Independent of the mode of administration 70-80 % of the nifedipine was eliminated via urine, 9/10 of this quantity being eliminated within the first day showing a half life of 4 to 5 hours. Upto 15 % are eliminated in the faeces uptill 4 days after oral or sublingual administration (48). In animals 50-70 % of the administered nifedipine is eliminated in urine and 30-40 % via faeces (47).

Nifedipine is completely metabolised in the human organism. The same metabolic breakdown is suggested in rats and dogs (47).

Only traces of unchanged nifedipine are eliminated through renal pathway. In Fig. 13 a metabolism scheme of nifedipine is presented (39). Metabolite II is present both in urine and in plasma (39). The metabolite 1 is only present in plasma (39). All metabolites are, when compared to nifedipine parent compound, pharmacologically inactive nifedipine derivatives (56). Unchanged nifedipine is present only in plasma. Metabolite III and the lacton-form are detected both in plasma and urine (39). The structures of all metabolites have been elucidated through spectroscopic techniques (27,31). The metabolites detected in

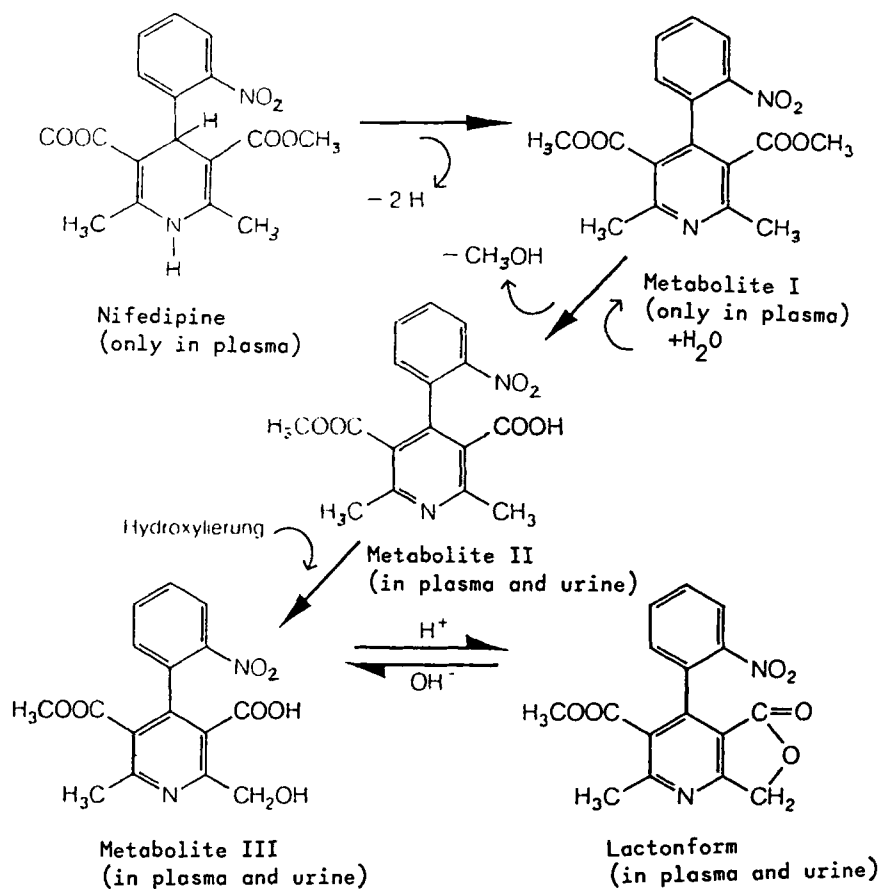


Fig. 13 (39)

Metabolism of Nifedipine

animal experiments could also be found in serum and urine of human beings (31,35,56).

Nifedipine is highly bound to protein and is a neutral compound of neither strongly lipophilic nor hydrophilic character. It is completely absorbed but undergoes significant, variable, first pass metabolism resulting in 45 % to 68 % absolute bioavailability (57). The initial metabolite observed in plasma is the pyridine analog of nifedipine formed by dehydrogenation resulting in aromatization (58).

The renal clearance of nifedipine is reported to be zero. More than 90 % of the metabolites are eliminated renally and are present in urine in 24 hours. The half-life of nifedipine capsules after oral and sublingual administration is given 4 hours, for nifedipine film tablets about 2 hours, for sustained-release formulations 6-12 hours and after i.v.-application about 2 hours (48).

On account of the reported short half-life of nifedipine of 2-3 hours no accumulation of the unchanged drug has been found in body tissues after long-time therapy. The metabolites of nifedipine are to large extent protein-bonded in plasma (47). Nifedipine is almost completely metabolised in the organism of the rat and dog. Nitropyridine derivative is the main metabolite detected in urine and the corresponding lactone metabolite is also present in urine (less than 5 %). Traces of the unchanged drug has been detected in the urine only after the administration of high doses of nifedipine (27).



Sublingual administration of 10 mg nifedipine was performed in four healthy volunteers. They were instructed to bite the capsule apart and keep the substance in the mouth for 5 minutes before swallowing. Plasma concentrations of nifedipine as well as the nitropyridine product as main metabolite were determined. Two persons had a fast absorption with maximum concentrations of 70-100 ng/ml nifedipine, reached within 30-60 minutes and two persons had a slower absorption with maximum concentrations of 20-40 ng/ml reached within 90-120 minutes (28). Plasma concentrations of nifedipine in patients with renal failure as well as in those with normal renal function were determined. Whereas the presence of renal failure did not influence the plasma concentrations, lower concentrations were found in the patients on haemodialysis than in the other groups (59).

In the human body nifedipine is rapidly oxidized enzymatically to its nitropyridine metabolite (48,22). This product is also formed in ultraviolet light, whereas the 2-nitroso derivative is formed in normal daylight. In the biotransformation process the ester moiety of the side chain can be hydrolysed and further oxidation can occur in the 2-methyl position. The first product is excreted in urine for about 60 % of the dose, whereas the second one is excreted for only 3-5 % of the dose (30). It has been shown by Kondo et al. (31) that unchanged nifedipine and or the nitropyridine derivative are excreted in urine in trace amounts (0.1 % of an oral dose). Plasma concentrations of nifedipine were monitored in two subjects. The absorption rates and the elimination profiles were significantly different between the two subjects (42).

The pharmacokinetic behaviour of new nifedipine preparations in humans was studied. Following a single dose of a 20 mg nifedipine sustained-release tablet to 12 healthy volunteers in a randomized two-fold cross-over trial, maximum plasma concentrations in the range of 21.5 to 73.9 ng/ml were found between 45 minutes and 2 hours. Minimum therapeutic plasma level of 10-15 ng/ml was maintained upto 8 to 10 hours. The relative bioavailabilities and important pharmacokinetic parameters were calculated. Terminal elimination half-lives were calculated to be 5 and 8 hours respectively (60).

Bioavailability of two preparations of soft gelatine capsules containing 10 mg nifedipine was performed. Mean peak plasma concentrations of  $121.2 \pm 47.3$  and  $104.5 \pm 32.9$  ng/ml were reached after about 30 minutes. Both plasma level curves were practically identical with the exception of the obtained absolute peak plasma concentrations. Some important pharmacokinetic parameters and the relative bioavailabilities of the preparations were calculated and compared statistically (61). In a randomized three-way crossover study with twelve volunteers the bioavailability and main pharmacokinetic parameters of three different galenic formulations of nifedipine (hard gelatine capsules with pellets, soft gelatine capsules with liquid nifedipine and a sustained-release tablet) were determined. Statistically significant differences for AUC,  $C_{max}$  and  $t_{max}$ -value were found between three preparations.  $C_{max}$ -values lie between 40-201 ng/ml and  $t_{max}$ -value between 0.48 - 1.96 hours. No accumulation of nifedipine in plasma occurred following multiple dosing (62). In another investigation the bioavailability parameters of different nife-

dipine sustained-release formulations, film tablets, hard gelatine capsules with pellets and nifedipine in solution were evaluated (43).

A bioequivalence study of 4 commercial nifedipine formulations was carried on. No therapeutic significant differences were found from the evaluated parameters AUC,  $C_{max}$ ,  $t_{max}$  among the commercial preparations (63). A review of nifedipine with focus on pharmacokinetic characteristics, available analytical methodologies and selected aspects of clinical pharmacology is presented (64). The pharmacokinetics of a sustained-action oral preparation of nifedipine (Adalat retard) following a single dose of 20 mg tablet was studied in 9 male volunteers and in one subject nifedipine was compared to the conventional capsule. Compared to conventional nifedipine capsule there was delay in peak concentration and much smaller range of peak plasma levels were observed (65).

The effects of Atenolol and Metoprolol on nifedipine plasma concentrations and the effects of nifedipine on beta blocker plasma levels were evaluated in 8 female subjects. No pharmacokinetic interaction between nifedipine and betablockers was found (66). Nifedipine and its metabolites were analysed in plasma of 6 volunteers after receiving both an i.v. injection (3.5 mg infusion over 4 min) and 2 new oral tablet formulations (20 and 30 mg). Oral administration of nifedipine was associated with a bioavailability of 0.43 and presence of its nitropyridine metabolite in the plasma. The nitropyridine metabolite was present only in trace amounts in plasma samples taken from subjects following i.v. administration. The peak plasma

concentration and AUC-time curve suggest that the nitropyridine analog is a major, first-pass metabolite of nifedipine (58).

The pharmacokinetics and pharmacological effects of nifedipine by i.v. injection of 0.015 mg/kg, 20 mg capsule and 20 mg sustained-action tablet were compared in 8 healthy subjects in a crossover design. After i.v. injection the elimination half-life was  $1.7 \pm 0.5$  h, systemic clearance was  $26.7 \pm 5.4$  l/h and volume of distribution was  $0.8 \pm 0.2$  l/kg. After capsule administration plasma concentration rose rapidly to a maximum of  $117 \pm 15$  ng/ml at a  $t_{\max}$  of  $1.4 \pm 0.5$  h. After the sustained-action tablet  $t_{\max}$  was  $4.2 \pm 0.7$  h and  $C_{\max}$  was  $26 \pm 10$  ng/ml. Bioavailability of nifedipine was  $56 \pm 25$  % after capsules and  $32 \pm 8$  % after tablets. There was a hyperbolic correlation between nifedipine plasma concentrations and changes in diastolic blood pressure, with a minimal effective concentration of about 15 ng/ml. It is concluded that nifedipine pharmacokinetic correlates directly with the effect on blood pressure and heart rate. Side effects from a high plasma concentration can be avoided with the tablet (67).

The pharmacokinetics of nifedipine was determined in 12 healthy adults after single doses of 10 mg orally and one mg i.v. administration. Nifedipine was eliminated after i.v. dosis with a half-life of  $1.77 \pm 0.25$  h and the total clearance was calculated to be  $0.62 \pm 0.09$  l/kg/h. With oral nifedipine dosage the elimination half-time was twice as long. Withing the group of subjects fast and slow absorption profiles for nifedipine were obtained. Bioavailability was ascertained with  $0.45 \pm 0.08$  (55).

Clinical efficacy and bioavailability of a sustained release nifedipine formulation containing 20 mg was studied. The tablets were administered twice daily to 30 patients suffering from coronary diseases and or hypertension. The product under study showed a satisfactory bioavailability when compared to the reference preparation (68). Bioavailability and bioequivalence of a new nifedipine preparation was tested in comparison to a reference preparation following a single dose administration of 5 mg soft gelatine capsule of each preparation to 12 healthy volunteers in a randomized, 2-fold cross-over trial. About 0.5 hour after administration peak plasma concentrations of about 110-120 ng/ml were reached, the mean terminal half-life being 1.5 h. The AUCs were, except for the negligible different peak concentrations, virtually identical. Both preparations were considered to be bioequivalent (69).

The effect of food on the bioavailability of nifedipine, 10 mg capsules was studied under fasting, after a low-fat meal and after a high-fat diet. The results indicated that the rate, but not the extent of nifedipine oral absorption is altered by food. The  $C_{max}$ - and  $t_{max}$ -values for fasting, low-fat and high-fat meals were 78.9, 42.2 and 58.7 ng/ml and 0.97, 1.89 and 1.07 hours respectively (70).

The plasma pharmacokinetics of nifedipine and the formation of its metabolites have been studied in volunteers under conditions which would affect the activity of the cytochrome p-450-system. Smoking does not contribute significantly to the variability in nifedipine pharmacokinetics. However the interaction between nifedipine and cimetidine, but not ranitidine, may be of clinical impor-

tance (71). The variability of 12 h plasma nifedipine concentrations in 64 hypertensive patients on long-term nifedipine retard 20 mg twice daily has been studied. A slightly skewed unimodal distribution with a modal concentration of 15-30 ng/ml was obtained. No relationship between 12 h plasma levels and debrisoquine hydroxylation phenotype was found (72).

The rate of protein binding of nifedipine, its main metabolite and other coronary-active substances was investigated by means of the ultracentrifugal method. Nifedipine is very strongly bound by the proteins of human serum, the  $\beta$ -value (percentage of bound substance) being between 91-98 %. The main metabolite of this substance, however, is bound to an extent of 54.5 %. There was no difference between the binding capacity of human and dog albumin (73).

The relative bioavailability of two nifedipine sustained-release preparations, pellets in hard gelatine capsules and film tablets was investigated in 10 volunteers in a cross-over method. The relative bioavailability of the test preparation was found to be an average of 106.6 % in comparison to the reference preparation (74). Variability in nifedipine pharmacokinetics and dynamics has been studied. The data obtained confirms the previous observations that the disposition of nifedipine is highly variable. The bimodal frequency distribution of the AUC clearly suggests that polymorphism exists with respect to disposition kinetics of nifedipine (75). The total systemic plasma clearance is in the order of 600-700 ml/min, indicating that elimination is dependent on hepatic blood

flow (58, 76). On the basis of the high systemic plasma clearance a systemic availability of 30-40 % and complete absorption can be assumed due to first-pass extraction in the liver. However in some studies mean systemic availability values of up to 75 % have been found (26). These results are most likely explained by the use of non-specific GLC assay, procedures, in which nifedipine is oxidised into its pyridine analogue during the analysis, so that the metabolites are co-analysed (32, 28, 31). In a recent study in which the assay method was more specific (33) a mean systemic availability of 45 % was found (55).

Nifedipine can be absorbed in the entire gastrointestinal tract depending on the mode of administration. If taken sublingually, part of the dose is already absorbed by the oral mucosa (77). The highest plasma concentration are usually observed after oral administration in the fasting state (78). Since the maximum plasma concentrations are reached within 20-40 minutes it could be concluded that rapid absorption takes place in upper parts of the small intestines (38). Nifedipine plasma concentrations are also measured after rectal application of suppositories (26).

## **12. Acknowledgement**

The author is indebted to Dr. Gerner and Dr. Haag, Bayer Leverkusen, Federal Republic of Germany for providing a lot of useful information and a number of spectra. Mrs. Monica Leschniewski has taken great pains in typing this manuscript. Mrs. Petra Groetsch kindly assisted in drawing the figures.

## References

- 1) A. Hantzsch, Justus Liebigs Ann. Chem., 215 1 (1882)
- 2) U. Eisner and J. Kuthan, Chemical Reviews 72 1 (1972)
- 3) B.M. Stout and A.I. Meyers, Chemical Review 82 223 (1982)
- 4) A.P. Phillips, J. Amer. Chem. Soc., 71 4003 (1949)
- 5) F. Bossert, W. Vater, Ger. offen. 1670827, Bayer AG from 20.3.1967; patent specification No. 1, 173, 862, The patent office London; date of application and filing complete specifications 20.3.1968.
- 6) H. Meyer, F. Bossert and E. Wehinger, Chemistry of Nifedipine in Treatment of Cardiovascular Diseases by Adalat, edited by R. Krebs, Schattauer Verlag, Stuttgart - New York 1986
- 6a) F. Bossert, H. Meyer and E. Wehinger, Angew. Chem. 93 755 (1981)
- 7) F. Bossert and W. Vater, Naturwissenschaften 58 578 (1971)
- 8) W. Vater and Coworkers, Arzneim.-Forsch./Drug Res. 22 1 (1972)
- 9) B. Loev and Coworkers, J. Med. Chem. 17 956 (1974)



- 10) Adalat Herzschtützendes Koronartherapeutikum, Kapseln/ Tabletten/Parenteralia, D 557/847968, Bayer Leverkusen, W. Germany, Februar 1986
- 11) The U.S. Pharmacopeia XXI, 21st edition, U.S.P. Convention, Rockville, Md. 120852, p. 733 (1985)
- 12) R. Mannhold, R. Rodenkirchen, R. Bayer and W. Haus, *Arzneim.-Forsch./Drug Res.* 34 407 (1984)
- 13) A.M. Triggle, E. Sheffer and D.Y. Triggle, *J. Med. Chem.* 23 1442 (1980)
- 14) H.W. Dibbern, UV and IR spectra of some important drugs, Editio Cantor, Aulendorf (1985)
- 15) F. Eiden, K. Braatz-Greeske, *DAZ* 123 2003 (1983)
- 16) S. Ebel, H. Schütz and A. Hornitschek, *Arzneim.-Forsch./Drug Res.* 28 2188 (1978)
- 16a) J. Kuhlmann, K.H. Graefe, K.D. Rämsch and R. Ziegler, *Clinical pharmacology in treatment of cardiovascular diseases by Adalat*, edited by R. Krebs, Schattauer Verlag, Stuttgart, New York 1986
- 17) K. Thoma and R. Klimek, *Pharm.Ind.* 47 207 (1985)
- 18) K. Thoma and R. Klimek, *Pharm. Ind.* 47 319 (1985)

- 19) M.-B. Bottorff, D.A. Kraves, R.G. Mcallister, R.L. Batenhorst and T.S. Foster, Am. J. Hosp. Pharm., 41 2068 (1984)
- 20) V. Gambaro, M. Coligara and E. Pesce, Bull. Chem. Farm. 124 13 (1985)
- 21) M. Sumnn, S.T.P. Pharma 2 299 (1986)
- 22) R. Testa, E. Dolfini, C. Reschiotto, C. Secchi and P.A. Biondi, Farmaco Ed. Prat. 34 463 (1979)
- 23) F.A. Tucker, P.S.B. Minty and G.A. MacGregor, J. Chromatogr. 342 193 (1985)
- 24) K. Thoma and R. Klimek, Deutsche Apotheker Zeitung, DAZ 120 1967 (1980)
- 25) K. Schloßmann, Arzneim.-Forsch./Drug Res. 22 60 (1972)
- 26) K.-D. Rämisch, Schwerpunkt Medizin 4 55 (1981)
- 27) H. Medenwald, K. Schloßmann and C. Wünsche, Arzneim.-Forsch./Drug Res. 22 53 (1972)
- 28) P. Jakobsen, O. Lederballe Pedersen and E. Mikkelsen, J. Chromatogr. 162 81 (1979)
- 29) K. Patzchke, K. Schloßmann and F.A. Horster, Ther. Ber., 47 197 (1975)

- 30) C.H. Kleinbloesem, J. Van Harten, P. Van Brummelen and D.D. Breimer, J. Chromatogr. 308 209 (1984)
- 31) S. Kondo, A. Kuchiki, K. Yamamoto, K. Akimoto, K. Takahashi, N. Awata and J. Sugimoto, Chem. Pharm. Bull. 28 1 (1980)
- 32) S. Higuchi and J. Shiobara, Biomed. Mass spectrom., 5 220 (1978)
- 33) S.R. Hamann and R.G. McAllister Jr., Clin. Chem. 29 158 (1983)
- 34) D. Lutz, G. Pabst, W. Dahmen, K.-H. Molz and H. Jaeger, Arzneim.-Forsch./Drug Res., 35 1840 (1985)
- 35) J. Dokladalova, J.A. Tykal, S.J. Coco, P.E. Durkee, G.T. Quercia and J.J. Korst, J. Chromatogr. 231 451 (1982)
- 36) M.T. Rosseel and M.G. Bogaert, J. Chromatogr. 279 675 (1983)
- 37) L.J. Lesko, A.K. Miller, R.L. Yeager and D.C. Chatterji, J. Chromatogr. Sci., 21 415 (1983)
- 38) K.D. Rämisch and J. Sommer, Hypertension (5) Suppl. 11 18 (1983)
- 39) K.D. Rämisch and R. Ziegler, 6th International Adalat Symposium, Geneva 18-20 April 1985. Monography published by Excerpta Medica, Amsterdam 23 (1986)

- 40) E. Zylber-Katz, G. Korn, L. Granit, M. Levy, Biopharm. Drug Dispos. 5 109 (1984)
- 41) Private Communication of Fa. Bayer, Leverkusen, W. Germany
- 42) K. Miyazaki, N. Kohri and T. Arita, J. Chromatogr. 310 219 (1984)
- 43) W. Stüber and Coworkers, Pharm.Ztg. 131 2963 (1986)
- 44) R. Herrmann, Pharm.Ztg. 130 2626 (1985)
- 45) P. Pietta, A. Rava and P. Biondi, J. Chromatogr. 210 516 (1981)
- 46) H. Suzuki, S. Fujiwara, S. Kondo and I. Sugimoto, J. Chromatogr. 341 341 (1985)
- 47) B. Duhm, W. Maul, H. Medenwald, K. Patzschke and L.A. Wegner, Arzneim.-Forsch./Drug Res. 22 42 (1972)
- 48) F.A. Horster, B. Duhm, W. Maul, H. Medenwald, K. Patzschke and L.A. Wegner, Arzneim.-Forsch./Drug Res. 22 330 (1972)
- 49) R.J. Gould, K.M. Murphy and S.H. Snyder, Life Science 33 2665 (1983)
- 50) Adalat, Kapseln, Tabletten, Parenteralia, P. 90, Bayer Leverkusen, West Germany, February 1986

- 51) J. Milovac, Z. Kopitar, M. Zorz and FD. Culig, Farm. Vestn. (Ljubljana, Yugoslavia) 36 21 (1985)
- 52) E. Parges, B. Schade and W. Ropte, Pharm. Ind. 47 77 (1985)
- 53) K. Patzschke, K. Schlossmann and F.A. Horster, Ther. Ber. 47 197 (1975)
- 54) K.-D. Rämisch, Schwerpunkt Medizin 4 55 (1981)
- 54a) P.W. Lückner, Adalat 10 mg SL tablets, Experts Expertise on the relative bioavailability of a new nifedipine formulations product-report (R 3049), Bayer AG, 1984
- 55) T.S. Foster, S.R. Hamann, V.R. Richards, P.J. Bryant, A. Graves and R.G. McAllister, J. Clin. Pharmacol. 23 161 (1983)
- 56) K. Schlossmann, H. Medenwald and H. Rosenkranz, 2nd Int. Adalat-Symposium Oktober 1974 in Amsterdam P. 33-39 (1975)
- 57) E.M. Sorkin, S.P. Clissold and R.N. Brogden, Drugs 30 182 (1985)
- 58) D.G. Waller, A.E. Renwick, B.S. Gruchy and C.F. George, British J. Clin. Pharmacol. 18 951 (1984)
- 59) M.G. Bogaert, M.T. Rosseel and J. Boelaert, Arzneim.-Forsch./Drug Res. 34 307 (1984)

- 60) D. Lutz, G. Pabst, W. Dahmen, K.-H. Molz and H. Jaeger, *Arzneim.-Forsch./Drug Res.* 35 1840 (1985)
- 61) W. Dahmen, G. Pabst, K.-H. Molz, D. Lutz and H. Jaeger, *Arzneim.-Forsch./Drug Res.* 35 1842 (1985)
- 62) G. Pabst, D. Lutz, K.-H. Molz, W. Dahmen and H. Jaeger, *Arzneim.-Forsch./Drug Res.* 36 256 (1986)
- 63) K.G. Bührens, W. Janssen and C. Hilgenstock, *Deutsche Apotheker Zeitung, DAZ* 125 1174 (1985)
- 64) S.R. Hamann, M.T. Piascik and R.G. McAllister *Biopharm. Drug Dispos.* 7 1 (1986)
- 65) F. Pasanisi, P.A. Meredith and J.L. Ried, *Int. J. Clin. Pharmacol. Res.* 5 63 (1985)
- 66) M.J. Kendall, D.B. Jack, S.J. Laughner, J. Lobo and S.R. Smith, *British J. Clin. Pharmacol.* 18 331 (1984)
- 67) C.H. Kleinbloesem, P. Van Brummelen, J.A. Van de Linde, P.Y. Voogd and D.D. Breimer, *Clin. Pharmacol. Ther.*, 35 742 (1984)
- 68) C. Piovetta, *Arzneim.-Forsch./Drug Res.* 37 832 (1987)
- 69) G. Achtert, R. Wisotzki, D. Lutz and H. Jaeger, *Arzneim.-Forsch./Drug Res.* 37 1296 (1987)
- 70) D.P. Reitberg, S.J. Love, G.T. Quercia and M.A. Zinny, *Clin. Pharmacol. Ther.*, 42 72 (1987)

- 71) A.G. Renwick, J. Le Vie, V.F. Challenor, D.G. Waller, B. Gruchy and C.F. George, Eur. J. Clin. Pharmacol. 32 351 (1987)
- 72) M. Beerahee, M.R. Wilkins, D.B. Jack, D.G. Beevers and M.J. Kendall, Eur. J. Clin. Pharmacol. 32 347 (1987)
- 73) H. Rosenkranz, K. Schlossmann and W. Scholtan, Arzneim.-Forsch./Drug Res. 24 455 (1974)
- 74) H. Blume, W. Stüber, G. Stenzhorn, E. Weber and R. Ding, Pharm.Ztg. 131 2534 (1986)
- 75) C.H. Kleinbloesem, P. van Brummelen, H. Faber, M. Danhof, N.P.E. Vermeulen and D.D. Breimer, Biochemical Pharmacology 33 3721 (1984)
- 76) R.E. Kates, Pharmacokinetic Properties, Drugs 25 113 (1982)
- 77) F.A. Horster, Pharmacokinetics of Nifedipine-14C in Man, 2nd International Adalat Symposium, Page 124, Springer, Berlin, Heidelberg, New York (1975)
- 78) S.J. Love, J. Yeh, J. Kann, M.J. Levit and D.P. Reitberg, Clin. Pharmacol. Ther. 37 209 (1985)

ANALYTICAL PROFILE OF  
PHYSOSTIGMINE SALICYLATE

*Farid J. Muhtadi and Seham S. El-Hawary*

Department of Pharmacognosy,  
College of Pharmacy, King Saud University,  
Riyadh, Saudi Arabia.



## PHYSOSTIGMINE SALICYLATE

CONTENTS**1. Description**

- 1.1 Nomenclature
- 1.2 Formulae
- 1.3 Molecular Weight
- 1.4 Elemental Composition
- 1.5 Appearance, Color and Odor
- 1.6 Loss on Drying

**2. Physical Properties**

- 2.1 Melting Range
- 2.2 Eutectic Temperature
- 2.3 Specific Optical Activity
- 2.4 Solubility
- 2.5 Dissociation Constant
- 2.6 Crystal Structure
- 2.7 X-Ray Powder Diffraction
- 2.8 Spectral Properties
  - 2.8.1 UV Spectrum
  - 2.8.2 IR Spectrum
  - 2.8.3 Nuclear Magnetic Resonance Spectra
  - 2.8.4 Mass spectrum

### **3. Isolation of Physostigmine**

#### **3.1 Preparation of Physostigmine Salicylate**

### **4. Synthesis of Physostigmine**

### **5. Biosynthesis of Physostigmine**

### **6. Drug Pharmacokinetics and Metabolism**

### **7. Drug Stability**

### **8. Methods of Analysis**

#### **8.1 Tests of Identification**

#### **8.2 Microcrystal Formation**

#### **8.3 Titrimetric Methods**

##### **8.3.1 Non-aqueous Titration**

##### **8.3.2 Potentiometric Titration**

##### **8.3.3 Iodometric Titration**

#### **8.4 Spectrophotometric Methods**

##### **8.4.1 Colorimetric Determination**

##### **8.4.2 UV Determination**

##### **8.4.3 Spectrofluorimetric**

##### **8.4.4 Phosphorescent Analysis**

#### **8.5 Enzymatic Methods**

#### **8.6 Chromatographic Methods**

##### **8.6.1 Paper Chromatography**

##### **8.6.2 TLC**

##### **8.6.3 HPLC**

##### **8.6.4 GLC**

#### **8.7 Other Methods.**

### **9. Therapeutic Uses**

## 1. Description

### 1.1 Nomenclature

#### 1.1.1 Chemical Names

(3a*S*-cis)-1,2,3,3a,8,8a-Hexahydro-1,3a,8-trimethylpyrrolo [2,3-*b*] indol-5-ol methylcarbamate (ester).

Pyrrolo [2,3-*b*] indol-5-ol, 1,2,3,3a,8,8a-hexahydro-1,3a,8-trimethyl-methylcarbamate (ester), 3a *S*-cis.

(3a*S*, 8a*R*)-1,2,3,3a,8,8a-hexahydro-1,3a,8-trimethylpyrrolo [2,3-*b*] indol-5-yl methylcarbamate salicylate.

1,2,3,3a,8,8a-Hexahydro-1,3a,8-trimethylpyrrolo-[2,3-*b*] indol-5-ol methylcarbamate (ester), monosalicylate.

Pyrrolo [2,3-*b*] indol-5-ol, 1,2,3,3a,8,8a hexahydro-1,3a,8-trimethyl-methylcarbamate (ester): (3a *S*-cis)-, mono(2-hydroxybenzoate).

#### 1.1.2 Generic Names

Physostigmine Salicylate; Eserine Salicylate; Physostol Salicylate.

#### 1.1.3 Trade Names for Physostigmine Salicylate

Antilirium (injection); Isopto (Ophthalmic solution, with chlorobutanol and sodium sulfite).

### 1.2 Formulae

#### 1.2.1 Empirical

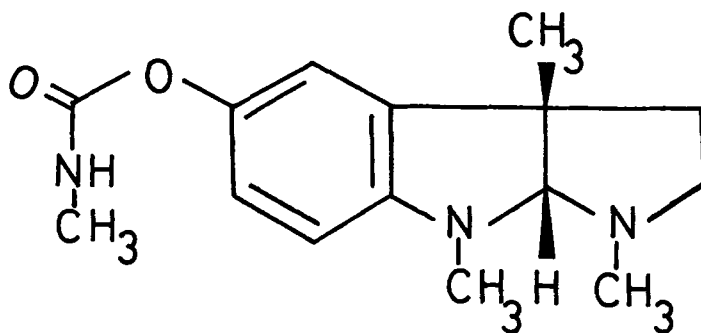
$C_{15}H_{21}N_3O_2$  (Physostigmine)

$C_{15}H_{21}N_3O_2 \cdot C_7H_6O_3$  (Physostigmine Salicylate)

$(C_{15}H_{21}N_3O_2)_2, H_2SO_4$  (Physostigmine Sulfate).

#### 1.2.2 Structural

Physostigmine is an indole alkaloid, having a urethane side chain.



It is the major alkaloid of Calabar bean "*Physostigma venenosum*" Balf., family Leguminosae (1).

The structure of physostigmine was deduced by Stedman and Barger in 1925(2) and has been confirmed by the total synthesis, which was first carried out by Julian and Pikel in 1935 (3) and later by other authors (4-7).

#### 1.2.3 CAS Registry Number

[57-47-6] Physostigmine.

[57-64-7] Physostigmine salicylate.

[64-47-1] Physostigmine sulfate.

#### 1.2.4 Wiswesser Line Notation (8)

T B556 EN GNT T & JBEG KOV M1

BQ (Physostigmine)

T B556 EN GNT T & JBEG KOV M1

& QVR BQ (Physostigmine salicylate)

#### 1.2.5 Absolute Configuration

The absolute configuration has been deduced from combinations of chemical degradations and correlations (9), O.R.D. studies of (-)-physostigmine with several related (-)-substances (9) and by the use of the nuclear Overhauser effect (NOE) in NMR spectroscopy (10) together with the determination of the crystal structure by measuring the three-dimensional intensity data on a computer-controlled four-circle diffractometer (11).

The absolute structure of (-)-physostigmine is shown above and a perspective view of it,

projected on the plane of  $N_5-N_7-C_9-C_{11}$  is presented in Fig. 1 (11).

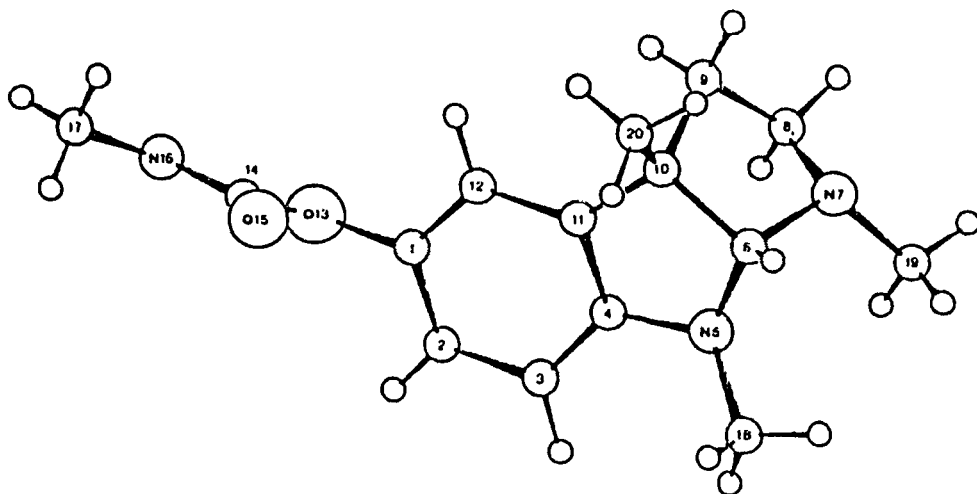


Fig. 1. The Absolute Configuration of (-)-Physostigmine

### 1.3 Molecular Weight

275.34	(Physostigmine)
413.46	(Physostigmine salicylate)
648.77	(Physostigmine sulfate)

### 1.4 Elemental Composition

C, 65.43%; H, 7.69%; N, 15.26%; O, 11.62%.  
(Physostigmine).

C, 63.91%; H, 6.58%; N, 10.16%; O, 19.35%.  
(Physostigmine salicylate).

### 1.5 Appearance, Color and Odor

Physostigmine crystallizes from ether or benzene as orthorhombic sphenoidal prisms or clusters of leaflets (12) or white odorless microcrystalline powder (13).

Colorless acicular crystals (14), or white or faintly yellow odorless powder (13), which gradually turn red on exposure to air and light (Physostigmine salicylate).

White odorless microcrystalline powder (Physostigmine sulfate).

### 1.6 Loss on Drying

Physostigmine salicylate when dried to constant weight at 100° to 105°, loses not more than 1.0% of its weight; use 0.1g (14).

## 2. Physical Properties

### 2.1 Melting Range

The following melting points are reported:

Physostigmine : 105-106° (12); not lower than 103° (13);

Physostigmine salicylate : 185-187° (12); at about 184° (13);

Physostigmine sulfate : 140° (after drying at 100°) (12); at about 143° (13).

### 2.2 Eutectic Temperature (15):

The eutectic temperature of physostigmine salicylate is recorded as follows:-

	Hot Bar Method	Microscope Hot Stage
Benzan.	141°	139°
Sal.	156°	156°

---

Benzan. = Benzanilide; Sal. acetaminosalol

### 2.3 Specific Optical Activity

$[\alpha]_D^{17} - 76^\circ$  (c=1.3 in chloroform);  $[\alpha]_D^{25} - 120^\circ$  (benzene) (12). For physostigmine.

Its specific rotation in aqueous solution is between  $-91^\circ$  and  $-94^\circ$  (14); For physostigmine salicylate. Specific optical rotation of about  $-92^\circ$ , determined on a 1% w/v solution (13). For physostigmine salicylate.

### 2.4 Solubility

Physostigmine is slightly soluble in water, freely soluble in alcohol, benzene, very soluble in chloroform, it is soluble in fixed oils (12,13).

Physostigmine salicylate is soluble in 90 parts of water and in 25 parts of ethanol (96%), very slightly

soluble in ether (14); 1 g is soluble in 75 ml water, 16 ml alcohol, 6 ml chloroform and about 250 ml ether (12,13).

One gram of physostigmine sulfate is soluble in 4 ml of water, 0.4 ml alcohol and about 1200 ml ether (12,13).

## 2.5 Dissociation Constant

$$K_1 \ 7.6 \times 10^{-7}; K_2 \ 5.7 \times 10^{-13} \quad (12).$$

$$pK_{a1} \ 6.12; pK_{a2} \ 12.24 \quad (13).$$

## 2.6 Crystal Structure

Crystals of physostigmine are orthorhombic, space group  $P2_1^2 2_1^2 2_1^2$  with  $a = 1458(1)$ ,  $b = 1435(1)$ ,  $c = 727.1(6)$  pm.

The values of  $R$  are 0.060 over the 1,520 significant reflexions and 0.097 over all 2,531 data (11).

The conformation of the molecule is shown in Fig. 1, the absolute configuration corresponds to that of the natural alkaloid. The two pyrrolidine rings are *cis*-fused, and that adjacent to the aromatic ring is practically flat, while the other is in the half-chair ( $C_2$ ) conformation with the two-fold axis passing through C8 and the center of the C6-C10 bond. Both ring nitrogen atoms are tetrahedral, but N5 is rather flattened and has more  $sp^2$  character, judging from the bond angles. The C18 methyl group is *cis* to the hydrogen on C6. The lone pair on N7 is *cis* to the hydrogen atom on C6. The carbamate group is planar, with the hydrogen on N16 *trans* to the keto oxygen. The torsion angle  $C_{12}-C_1-O_{13}-C_{14}$  ranges  $-30^\circ$  to  $150^\circ$  and  $+30^\circ$  to  $+150^\circ$  (upon examination of CPK space-filling atomic models) (11).

## 2.7 X-ray Powder Diffraction

The X-ray diffraction pattern of physostigmine salicylate was determined on a Philips X-ray diffraction spectrogoniometer fitted with PW 1730 generator. Radiation was provided by a copper target (Cu anode 2000 w). High intensity X-ray tube operated at 40 KV and 30 MV was used. The monochromator was a single

curved crystal one ( $\gamma$ -1.54180Å). The unit was equipped with Philips PM 8210 printing recorder and digital printer. Divergence slit and the receiving slit were 1°. The scanning speed of the goniometer used was 0.02 2 $\theta$  second. The lower level and the upper one of the signal control were 35 and 75 respectively.

The X-ray pattern of physostigmine salicylate is presented in Fig. 2. Interplanar distance and relative intensity are placed in table 1.

Table 1 : X-Ray Diffraction Pattern of Physostigmine Salicylate

d(Å°)	I/I <sub>0</sub>	d(Å°)	I/I <sub>0</sub>	d(Å°)	I/I <sub>0</sub>
12.55	9.7	4.45	19.7	3.15	12.4
10.04	10.4	4.35	19.0	3.07	18.3
9.65	15.8	4.25	11.5	2.83	12.0
7.42	47.1	3.90	31.5	2.75	10.8
7.02	19.7	3.76	37.4	2.74	10.3
5.96	32.2	3.69	20.6	2.69 51	11.9
5.54	33.5	3.64	19.8		
5.31	18.1	3.49	33.4		
4.99	15.8	3.46	49.0		
4.84	17.0	3.39	10.5		
4.68	56.5	3.26	15.2		
4.55	100.0	3.17	11.8		

d = interplanar distance, I/I<sub>0</sub> = relative intensity (based on highest intensity of 100).



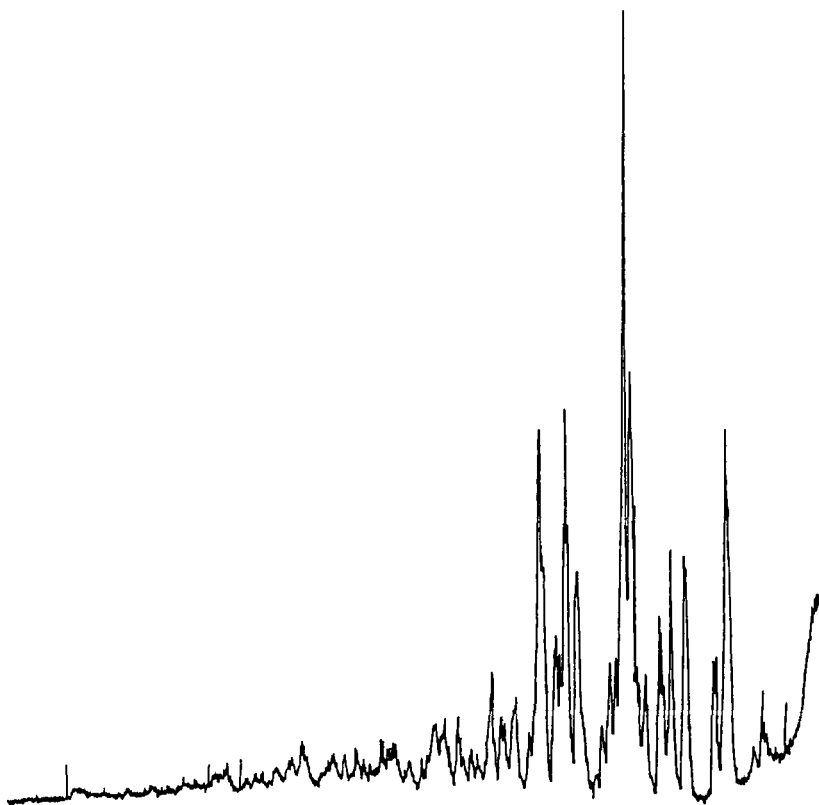


Fig. 2 : The X-Ray Diffraction Pattern of Physostigmine Salicylate.

## 2.8 Spectral Properties

### 2.8.1 Ultraviolet Spectrum

The UV spectrum of physostigmine salicylate in methanol (Fig. 3) was scanned from 200 to 400 nm using a Pye-Unicum SP 8-100 Spectrometer. Physostigmine salicylate exhibited the following UV data (Table 2).

Table 2 : UV Characteristics of Physostigmine Salicylate.

<u><math>\lambda_{\text{max. nm}}</math></u>	<u><math>\log \epsilon</math></u>	<u><math>A(1\%, 1\text{cm})</math></u>
239	4.09	297
252	4.04	266
303	3.78	146

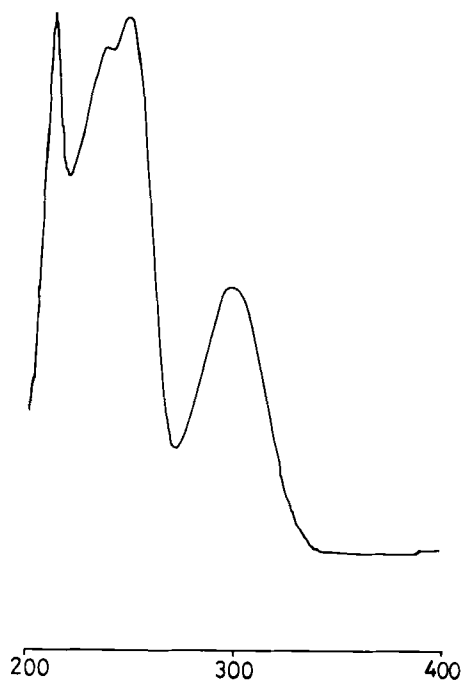


Fig. 3: The UV Spectrum of Physostigmine Salicylate in MeOH.

Other reported UV data for physostigmine are as follows:-

	<u>Solvent</u>	<u><math>\lambda_{\text{max}}</math>.nm</u>	<u>Ref.</u>
Physostigmine	0.2NH <sub>2</sub> SO <sub>4</sub>	246(E 1%, 1 cm=390), 302(E1%, 1cm =96).	(16)
	Methanol	253( $\epsilon$ 12700), 310( $\epsilon$ 3010).	(8)
Physostigmine Salicylate	Methanol	240( $\epsilon$ 25100), 300( $\epsilon$ 11200).	(8)

### 2.8.2 Infrared Spectrum

The IR spectrum of physostigmine salicylate as KBr-disc (Fig. 4) was recorded on a Perkin Elmer 580B Infrared Spectrophotometer to which an infrared data station is attached. The structural assignments of physostigmine have been correlated with the following frequencies (Table 3).

Table 3 : IR Characteristics of Physostigmine Salicylate.

<u>Frequency cm<sup>-1</sup></u>	<u>Assignment</u>
3310	NH stretch
2960, 2935	CH stretch
1745	$\begin{array}{c} \text{O} \\ \parallel \\ \text{-C-O-} \end{array}$ (aromatic ester)
1634	$\begin{array}{c} \text{O} \\ \parallel \\ \text{HN-C-} \end{array}$ (amide)
1595	C=C (aromatic)
1489, 1465	CH bending vibrations
1089, 1056, 1030	C-N vibration (aliphatic)
755	Monosubstituted aromatics

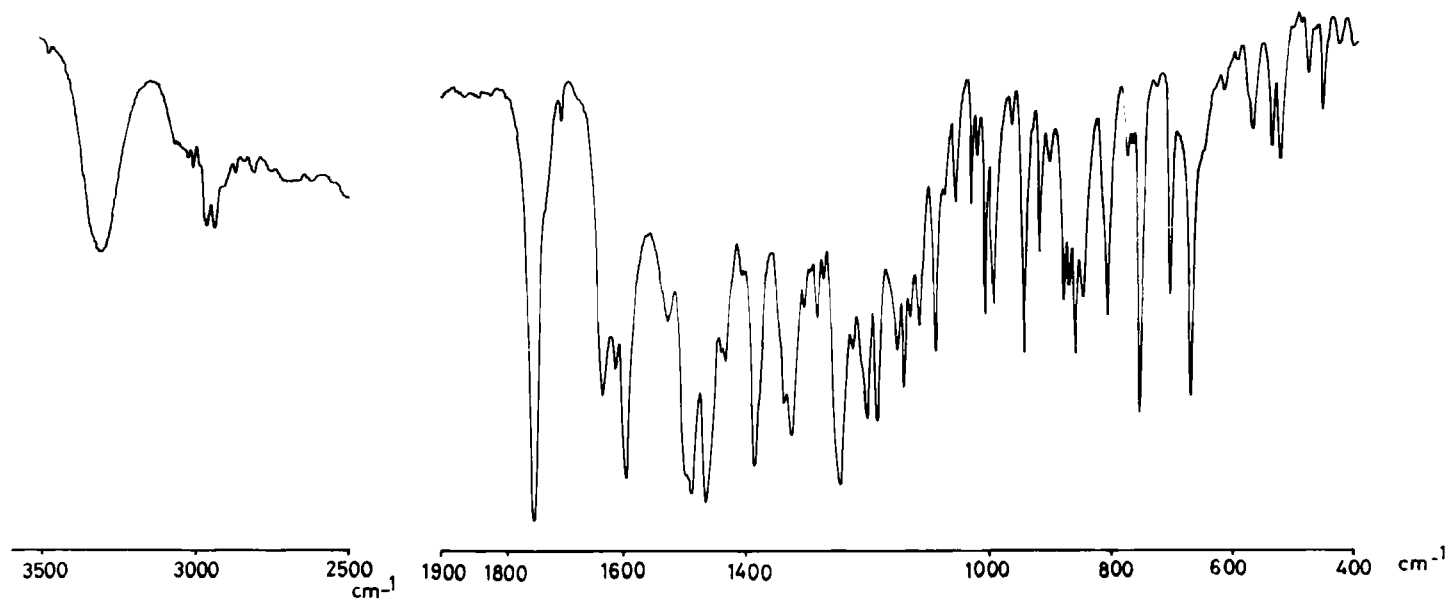


Fig. 4: The IR Spectrum of Physostigmine Salicylate in KBr disc.



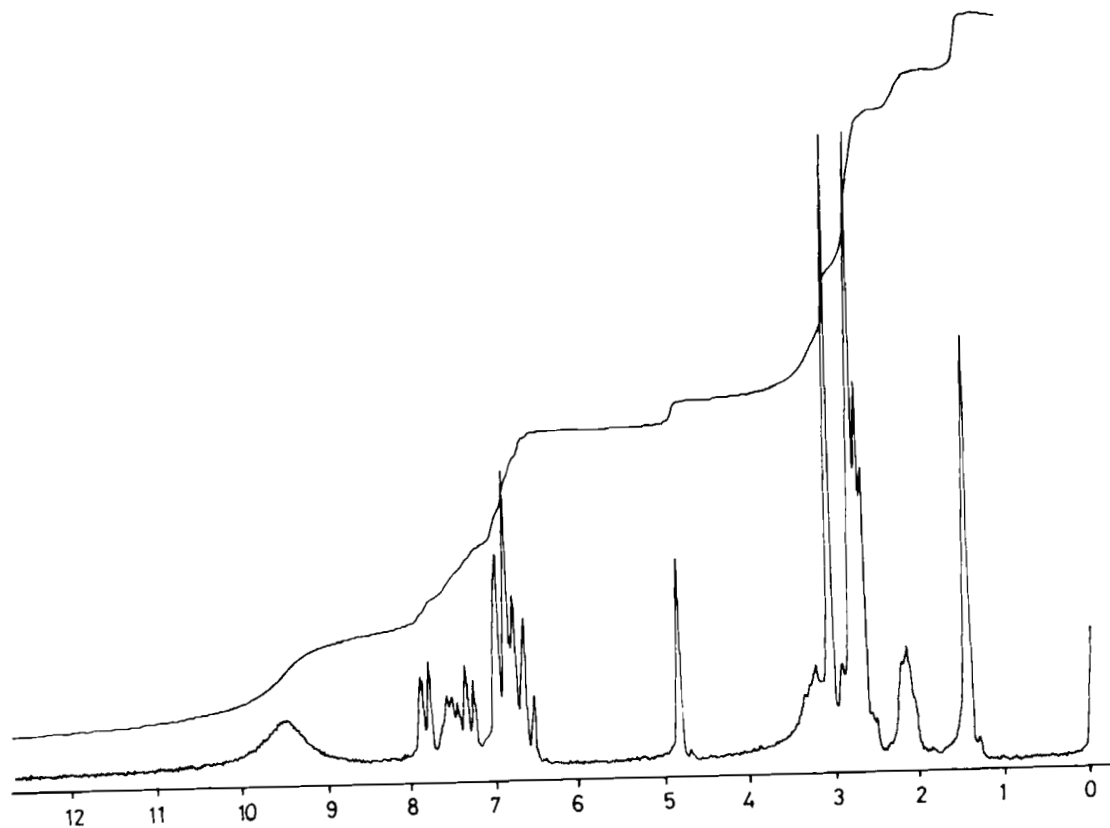


Fig.5(a) : The  $^1\text{H}$ -NMR Spectrum of Physostigmine Salicylate in  $\text{DMSO-d}_6$ .

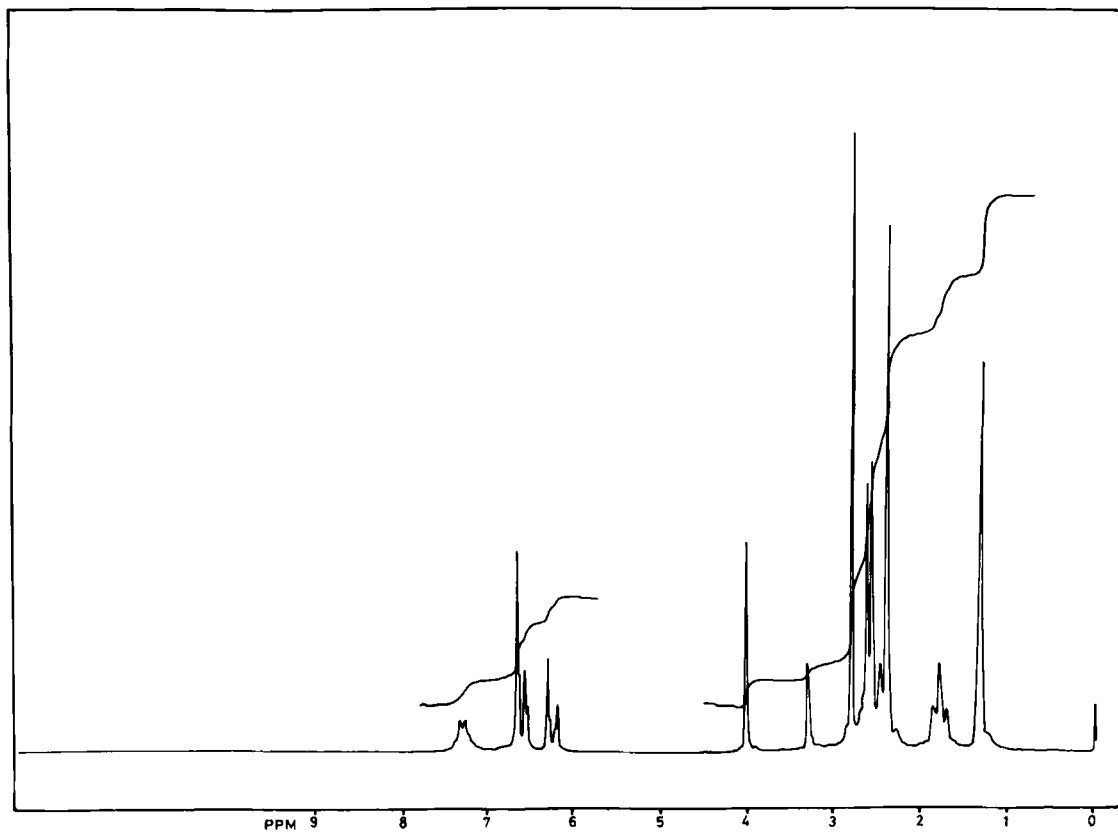


Fig.5(b) : The  $^1\text{H}$ -NMR Spectrum of Physostigmine in  $\text{DMSO-d}_6$ .

Chemical Shift (ppm)	Assignment	Multiplicity
6.85	4H } 5H } (aromatics) 6H }	singlet
6.77		A-B pair of doublets δ6.43, 6.81
6.49		
7.52	7H (NH)	quartet (D <sub>2</sub> O exchangeable)
2.73	8H (N-CH <sub>3</sub> )	doublet (sharpened into singlet upon D <sub>2</sub> O)
2.90	9H (N-CH <sub>3</sub> )	singlet
2.46	10H (N-CH <sub>3</sub> )	singlet
1.35	11H (C-CH <sub>3</sub> )	singlet

Other <sup>1</sup>H-NMR data for physostigmine have been reported (8,10,17).

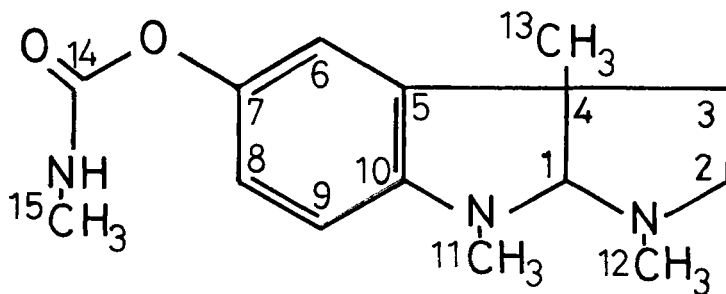
#### 2.8.3.2 <sup>13</sup>C-NMR Spectra

The <sup>13</sup>C-NMR completely decoupled and off-resonance spectra of physostigmine & its salicylate were recorded over 5000 Hz using a Varian FT 80 A Spectrometer. The alkaloid and salt were dissolved in DMSO-d<sub>6</sub> using a 10 mm sample tube and TMS as an internal standard at an ambient.

The spectra are presented in Fig. 6 and Fig. 7 respectively.

The carbon chemical shifts were assigned on the basis of the chemical shift theory and off-resonance splitting pattern (Table 5).

Assignment of all <sup>15</sup>C of physostigmine (Table 5) are consistent with those reported by Robonison et al (18).





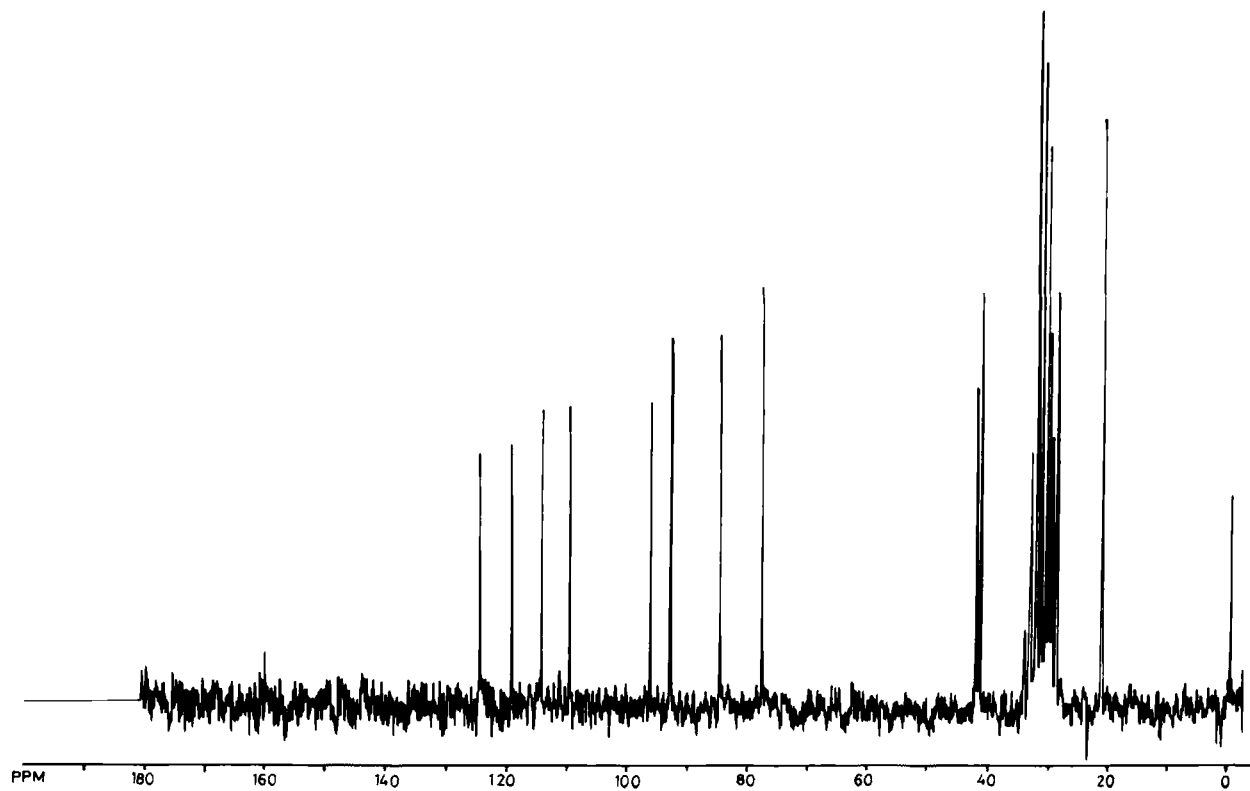


Fig. 6: The  $^{13}\text{C}$  - NMR decoupled Spectrum of Physostigmine in  $\text{DMSO-d}_6$ .

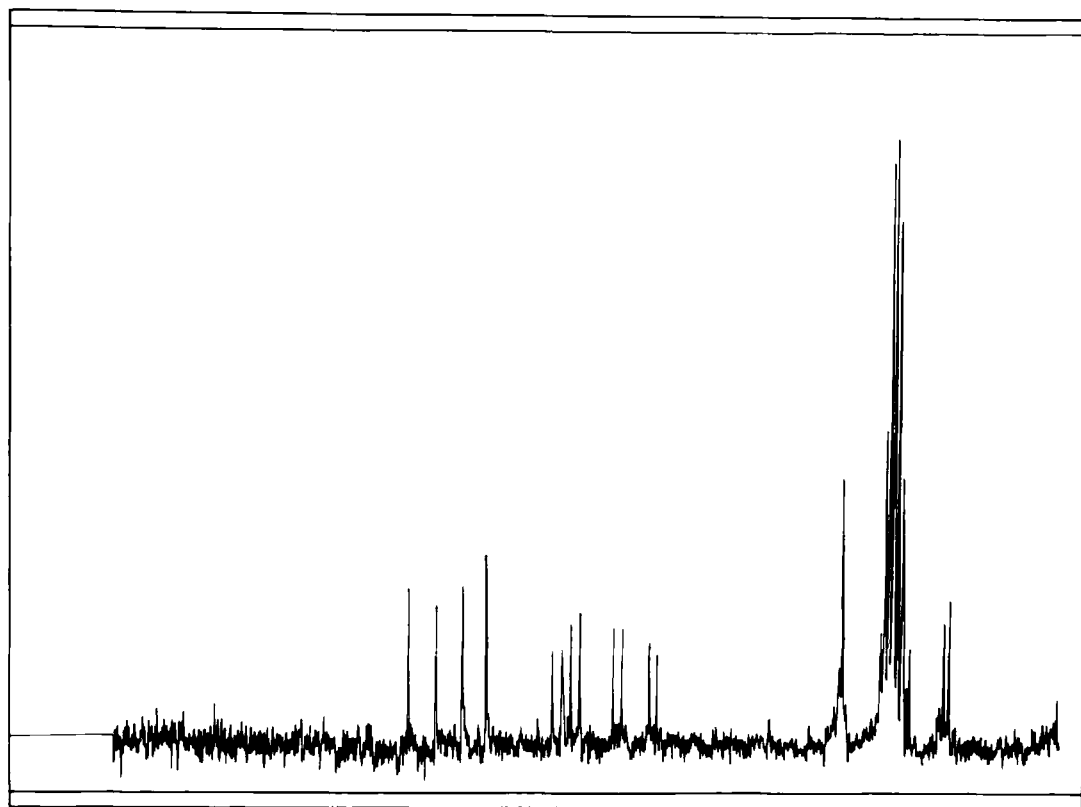


Fig. 7: The  $^{13}\text{C}$  - NMR off-resonance Spectrum of Physostigmine in  $\text{DMSO-d}_6$ .

Table 5. Carbon Chemical Shifts of Physostigmine

Carbon	Chemical Shifts [ppm]	Carbon	Chemical Shifts [ppm]
C <sub>1</sub>	97.18 (d)	C <sub>9</sub>	106.0 (d)
C <sub>2</sub>	52.50 (t)	C <sub>10</sub>	142.83 (s)
C <sub>3</sub>	40.47 (t)	C <sub>11</sub>	38.40 (q)
C <sub>4</sub>	51.90 (s)	C <sub>12</sub>	36.38 (q)
C <sub>5</sub>	137.16 (s)	C <sub>13</sub>	26.96 (q)
C <sub>6</sub>	116.15 (d)	C <sub>14</sub>	155.66 (s)
C <sub>7</sub>	149.10 (s)	C <sub>15</sub>	26.96 (q)
C <sub>8</sub>	120.28 (d)	-	

s = singlet; d = doublet; t = triplet;  
q = quartet.

Other <sup>13</sup>C-NMR data have also been reported (17,18,19).

#### 2.8.4 Mass Spectrum

The mass spectrum of physostigmine is presented in Fig. 8. This spectrum was obtained by electron impact ionization on an E.I. Finnigan model 300 (70 eV ionization potential) with INCOS data system.

The spectrum was scanned to the mass 300 amu. It shows a molecular ion peak M<sup>+</sup> at m/e 275. The base peak is at m/e 160.

The most prominent fragments and their relative intensities (RI) are listed in table 6.

Table 6. Mass Fragments of Physostigmine

m/e	RI	m/e	RI	m/e	RI
275	8.8	188	10.9	173	17.1
218	53.4	186	9.2	162	21.0
217	13.2	175	21.6	161	80.2
203	9.2	174	87.1	160	100.0

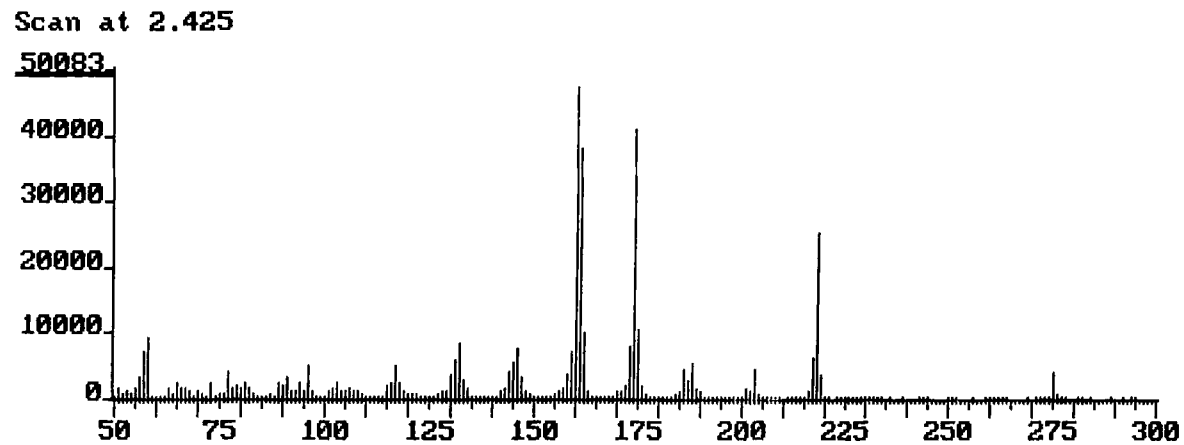
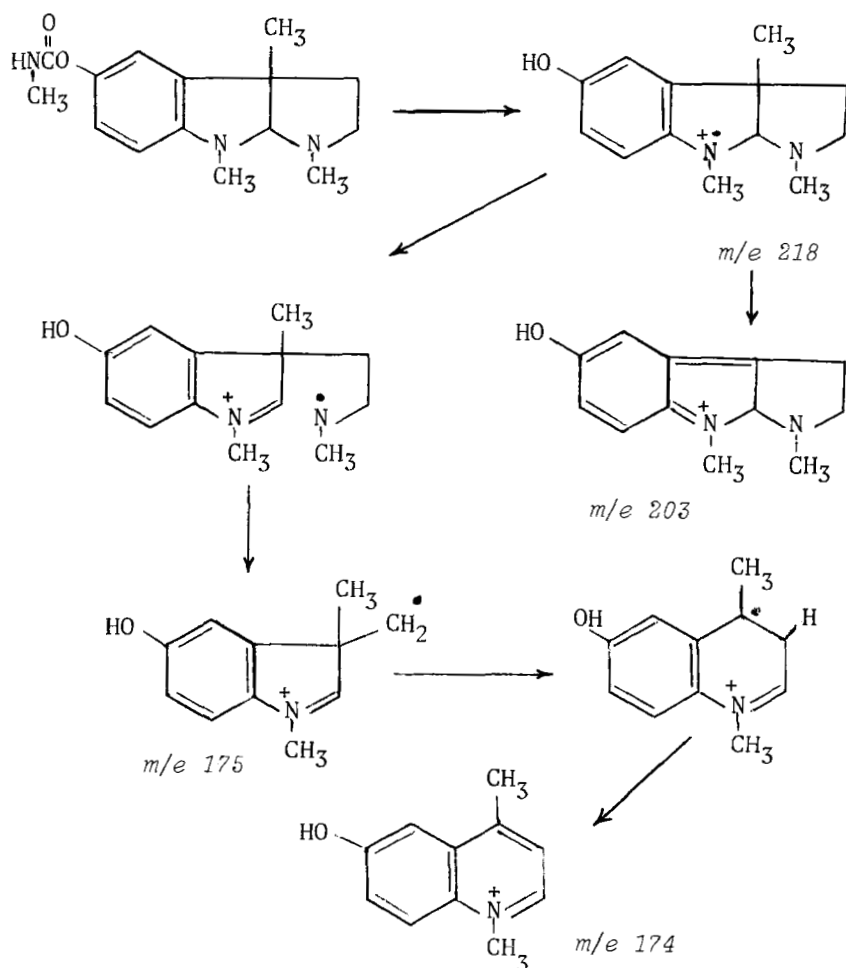


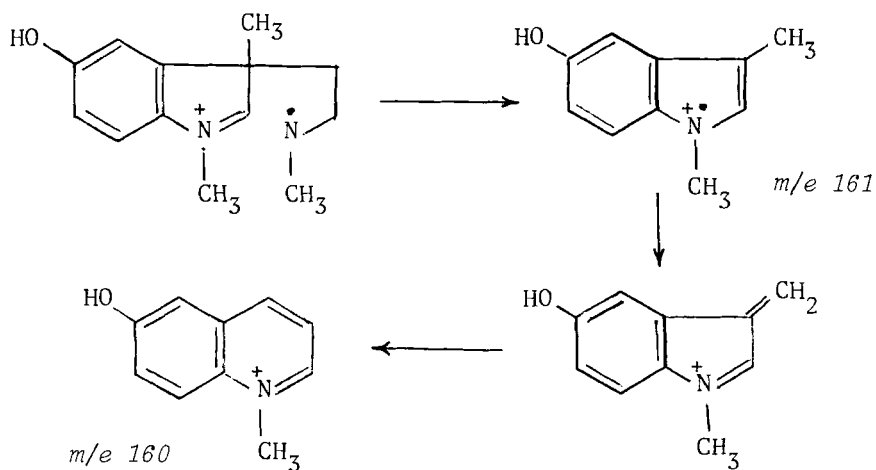
Fig. 8: The Mass Spectrum of Physostigmine.

m/e	RI	m/e	RI	m/e	RI
159	15.2	132	17.5	77	8.3
146	15.9	131	11.9	58	19.6
145	11.6	117	10.3	57	15.3
144	8.2	96	10.0		

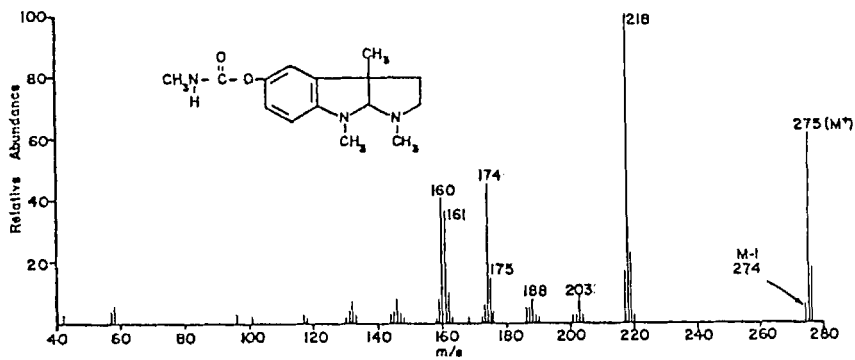
The fragmentation pattern of physostigmine has been interpreted by two groups (20,21,22), both concluding that the decomposition paths are predominantly controlled by the formation of relatively stable aromatic ions as it is evident from the following Fig. 9.

Fig. 9. Mass Fragmentation of Physostigmine





Other mass spectral data for physostigmine have also been reported (20-22). The following mass spectrum was reported by Djerassi et al. (22).



### 3. Isolation of Physostigmine

*Physostigmine occurs in Calabar bean to the extent of 0.15-0.3% (1).*

The powdered seed material is extracted by continuous percolation with hot alcohol. After extraction, the solvent is removed by distillation. Addition of water to the residue and separation of the floating fat layer are followed by alkalization of the aqueous liquid with sodium carbonate and repeated extraction with ether.

The ether extract is then concentrated to a small volume and washed repeatedly with 5% sulfuric acid until washings just become acidic. The aqueous acid solution is separated, rendered alkaline and the precipitated physostigmine is collected, dried and crystallized (23).

#### 3.1 Preparation of Physostigmine Salicylate

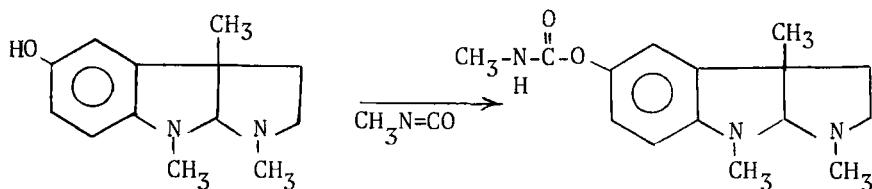
The salicylate salt is prepared by adding 2 parts of physostigmine to a solution of 1 part of salicylic acid in 35 parts of boiling distilled water and allowing the salt to crystallize on cooling (24).

In another procedure, an aqueous solution of physostigmine sulfate is added to a saturated solution of sodium salicylate, thereupon crystalline physostigmine salicylate is precipitated out, collected and dried (23).

### 4. Synthesis of Physostigmine

#### 4.1 Partial Synthesis

(-) Eseroline can be converted into (-)-physostigmine by treatment of the former with methyl isocyanate (25).



#### 4.2 Total Synthesis

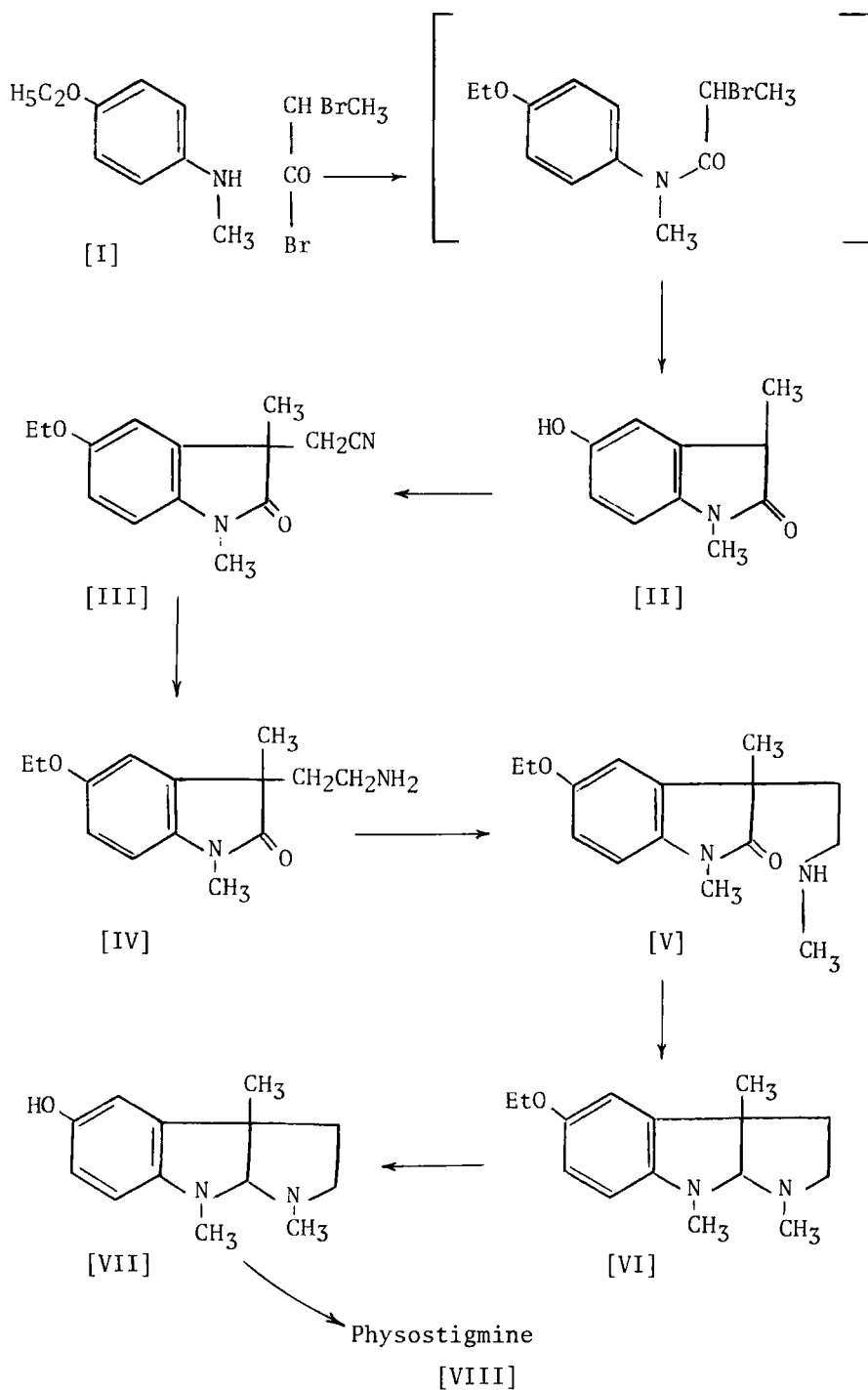
*The first total synthesis of physostigmine was achieved in 1935 by Julian and Píkl (3), since then several syntheses have been reported (4-7). The first synthesis is presented in scheme I.*

N-methylphenetidine [I] was condensed with  $\alpha$ -bromopropionyl bromide to give the amide adduct which when treated with aluminium chloride underwent cyclization, accompanied by loss of the ethyl group to give 1,3-dimethyl-5-hydroxyoxindole [II]. This was ethylated by treatment with ethyl sulfate and condensed with chloroacetonitrile in the presence of sodium ethoxide afforded the nitrile 1,3-dimethyl-5-ethoxyoxindolyl-3-acetonitrile [III]. Catalytic hydrogenation of [III] furnished the primary amine 1,3-dimethyl-5-ethoxy-3 $\beta$ -aminoethyloxindole [IV]. The latter upon condensation with benzaldehyde, quaternization of the resulting Schiff's base with methyl iodide and subsequent hydrolysis (Decker's method (26)) afforded the secondary amine 1,3-dimethyl-5-ethoxy-3 $\beta$ -methyl aminoethyloxindole [V] which was resolved by using optically active acids. Reductive cyclization of the (-) form of [V] with sodium and alcohol yielded (-)-eserethole [VI]. The latter was refluxed in light petroleum with aluminium chloride it yielded (-)-eseroline [VII] (identical with (-)-eseroline produced from (-)-physostigmine). (-)-Physostigmine [VIII] was then produced by treating [VII] with methyl isocyanate and applying the Polonovski and Nitzberg's method (25).

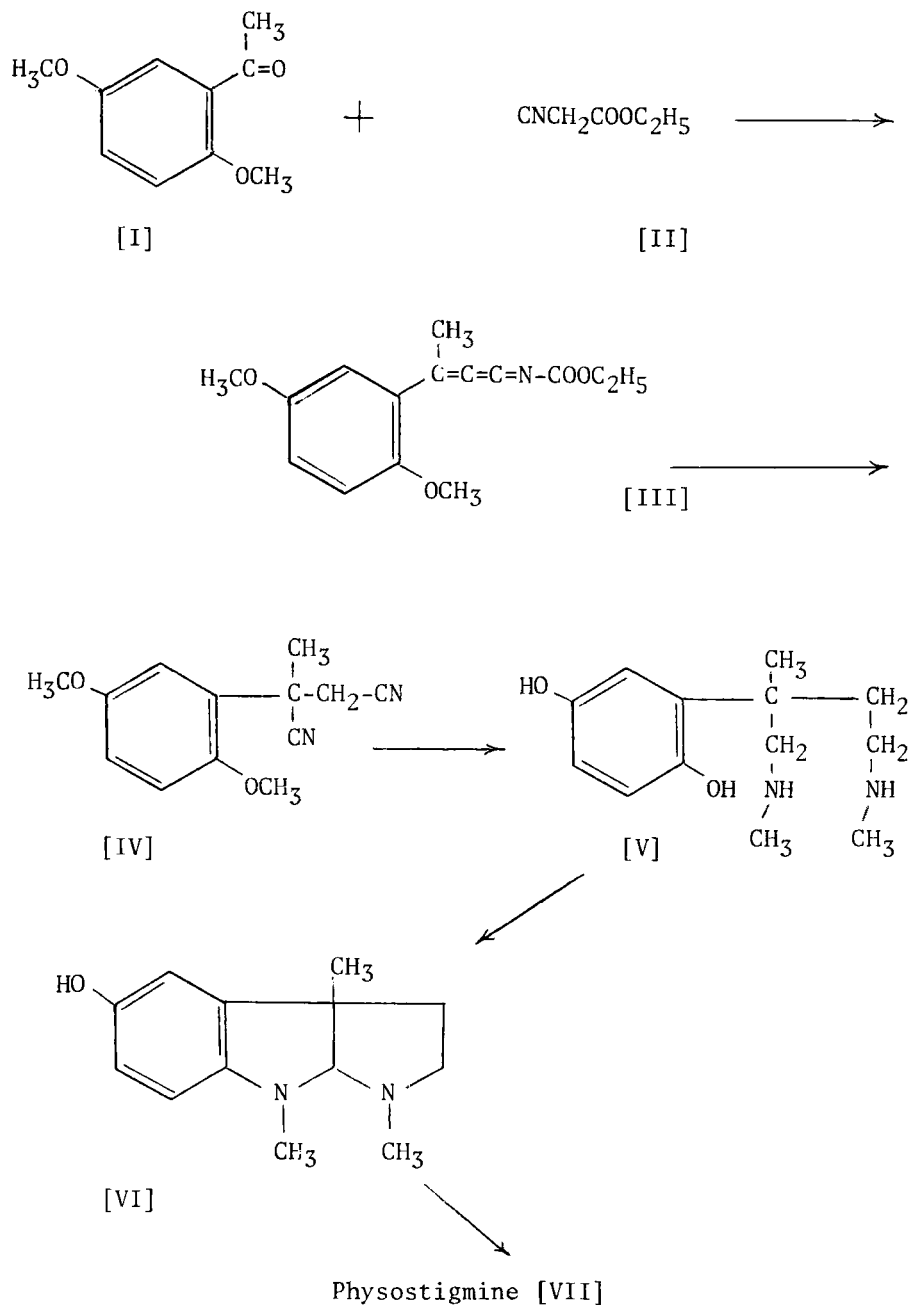
*The second total synthesis of physostigmine has been reported by Harley-Mason and Jackson (4). It is presented in scheme II.*

2,5-dimethoxyacetophenone [I] was condensed with ethyl cyanoacetate [II] by the procedure devised by Cope, Hofman, Wyckoff and Hardenburgh (27) to give ethyl-1-cyano-2-(2,5-dimethoxyphenyl) crotonate [III]. This was converted by treatment with potassium cyanide into  $\alpha$ -(2,5-dimethoxyphenyl)- $\alpha$ -methylsuccino nitrile [IV]. Hydrogenation of [IV] over platinum oxide in the presence of hydrochloric acid, yielded 2-(2,5-dimethoxyphenyl)-2-methylbutane-1,4-diamine [V]. The latter was oxidized with potassium ferricyanide to give ( $\pm$ )-eseroline [VI]. Identity was confirmed by ethylation of [VI] with ethyl-toluene-p-sulfonate to afford ( $\pm$ )-eserethole. Since



*Scheme I : The First Synthesis of Physostigmine*

*Scheme II: The Second Synthesis of Physostigmine*



( $\pm$ )-eserethole has earlier been resolved and converted into (-)-physostigmine [VII] ( 25 ), this method constituted a brief formal total synthesis of the natural alkaloid.

*A further synthetic approach to the physostigmine ring system has been developed (5). This approach is presented in scheme III ( 28 ) and constitutes the third synthesis of physostigmine.*

Ethyl-2-formylbutyrate *unsym*-N-methylphenylhydrazone [I] was indolized under Fischer conditions ( 29 ) to give [II] which was converted into [III] by alkaline hydrolysis. [III] was treated in methanol with methylamine at 50° to afford [IV]. The latter was reduced with lithium aluminium hydride to give ( $\pm$ )-desoxy-eseroline [V].

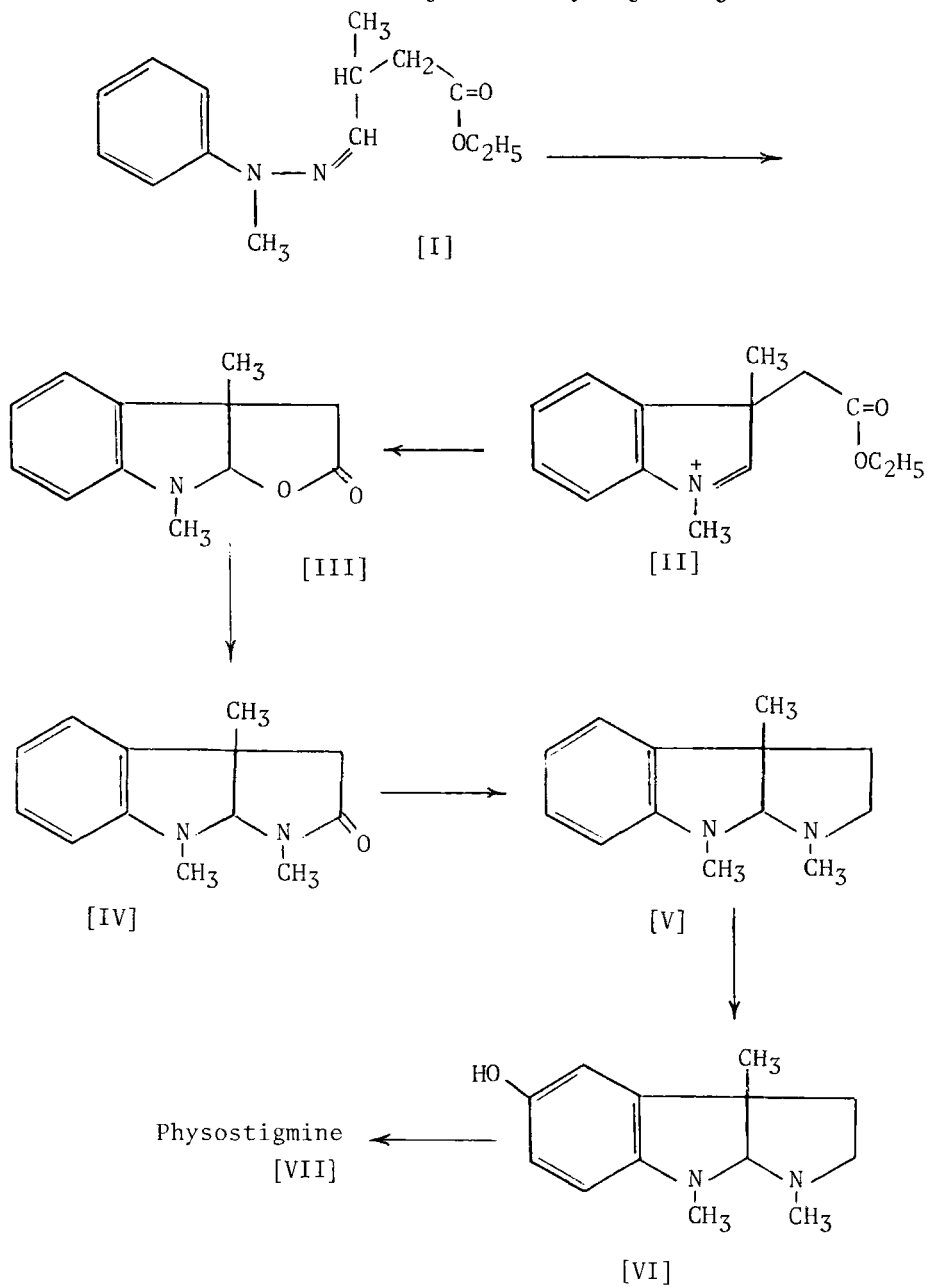
If [V] can be converted into ( $\pm$ )-eseroline [VI] by oxidation, (-)-physostigmine can then be produced as previously described.

*One of the most recent syntheses of the physostigmine ring system has been accomplished by Ikeda et al (6). This procedure is presented in scheme IV and constitutes the fourth total synthesis of physostigmine.*

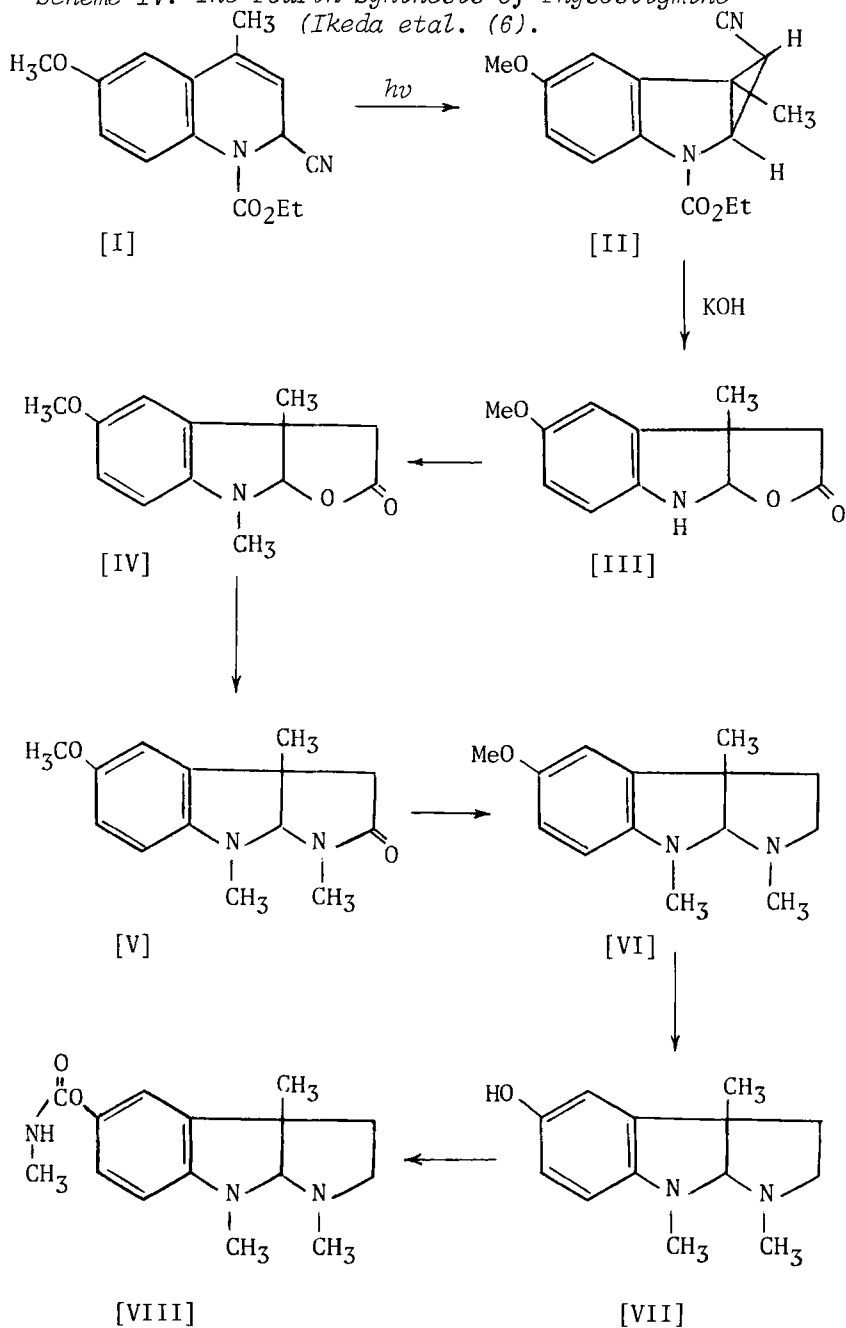
The methoxy derivative of ethyl-2-cyano-1,2-dihydroquinoline-1-carboxylate (Reissert compound) [I] was irradiated ( 30 ) to give the methoxy derivative of ethyl-1-cyano-1,1a,2,6b-tetrahydrocycloprop [b] indol-2-carboxylate [II] (photochemical synthesis). This cycloprop [b] indole [II] was treated with 10% potassium hydroxide and heated in a sealed tube at 120-130°C for 3.5 h. to produce the methoxy derivative of 3,3a,8,8a-tetrahydro-3a-methylfuro [2,3-b] indol-2-one [III].

[III] was treated with methyl iodide in acetone and heated in a sealed tube at 60°C for 4.5 h. to give 3,3a,8,8a-tetrahydro-5-methoxy-3a, 8-dimethylfuro [2,3-b]-indol-2-one [IV]. This was reacted with methylamine in ethanol to afford 3,3a,8,8a-tetrahydro-5-methoxy-1,3a,8-trimethylpyrrolo [2,3-b] indol-2-one [V]. The resulting pyrroloindole [V] was reduced with a solution of lithium aluminium hydride in dry tetrahydrofuran to yield ( $\pm$ )-esermethol (eseroline methyl ether) [VI] in quantitative yield (identical with an authentic sample prepared from physostigmine). Esermethole was then demethylated into ( $\pm$ )-eseroline [VII] which can be converted into physostigmine [VIII] by the standard procedure ( 25 ).

*Scheme III. The Third Synthesis of Physostigmine*

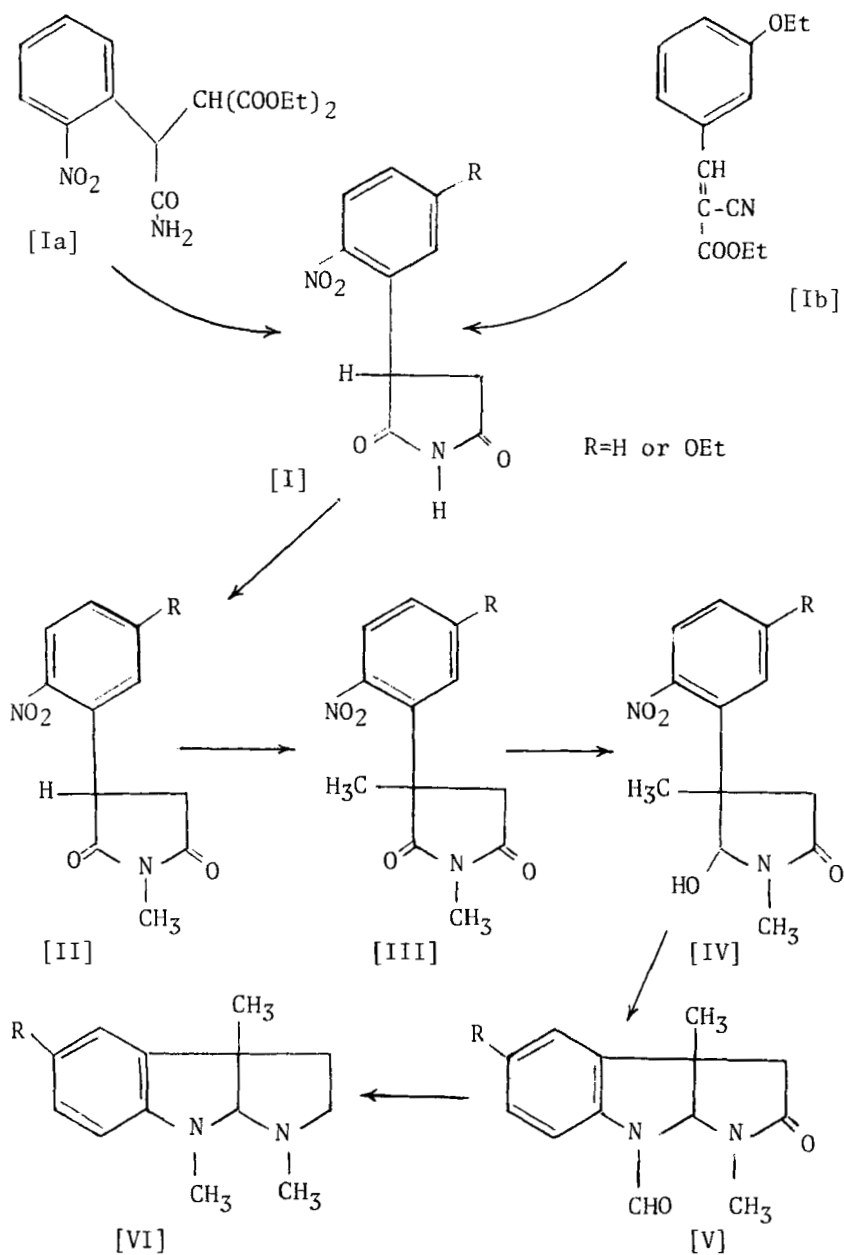


Scheme IV: The Fourth Synthesis of Physostigmine (Ikeda et al. (6).



*Physostigmine ring system has been constructed by other recent approach which was reported by Wijnberg and Speckamp (7). This synthesis is illustrated in scheme V.*

In this synthesis ( $\pm$ )-desoxyeseroline [VI] (R=H) was constructed from diethyl- $\alpha$ -carbamoyl- $\alpha$ -(2-nitrobenzyl) malonate [Ia]. A mixture of [Ia], sodium chloride and water in DMSO was heated for 7 hr. at 148°C, where cyclization occurred to give 3-(2-nitrophenyl succinimide [I] (R=H). [I] was N-methylated to afford 1-methyl-3-(2-nitrophenyl)-succinimide [II] (R=H). In this synthesis, also ( $\pm$ )-eserethole [VI] (R=OEt) was synthesized from ethyl- $\alpha$ -cyano-3-ethoxy cinnamate [Ib]. A mixture of [Ib] and KCN in methanol was refluxed for 30 minutes to yield 1-methyl-3-(3-ethoxyphenyl)-succinimide which was treated in acetic acid with fuming nitric acid to give 1-methyl-3-(2-nitro-5-ethoxyphenyl)-succinimide [II] (R=OEt). The latter was subjected to methylation with methyl iodide in the presence of potassium carbonate to yield either 1,3-dimethyl-3-(2-nitrophenyl)-succinimide [III] (R=H), or 1,3-dimethyl-3-(2-nitro-5-ethoxyphenyl)-succinimide [III] (R=OEt). Compound [III] was reduced with a mixture of THF and Na BH<sub>4</sub> in ethanol to give either 1,4-dimethyl-4-(2-nitrophenyl)-5-hydroxy-2-pyrrolidinone [IV] (R=H), or 1,4-dimethyl-4-(2-nitro-5-ethoxyphenyl)-5-hydroxy-2-pyrrolidinone [IV] (R=OEt). This was hydrogenated over 10% Pd/C where cyclization occurred to produce 1,3a-dimethyl-8-formyl-3,3a,8,8a-tetrahydropyrrolo [2,3-b] indol-2-one [V] (R=H), or 1,3a-dimethyl-5-ethoxy-8-formyl-3,3a,8,8a-tetrahydropyrrolo [2,3-b] indol-2-one [V] (R=OEt). Desoxyeseroline [VI] (R=H) was then produced by the reduction of [V] (R=H) with LAH, or eserethole [VI] (R=OEt) was obtained from [V] (R=OEt) with the same treatment.

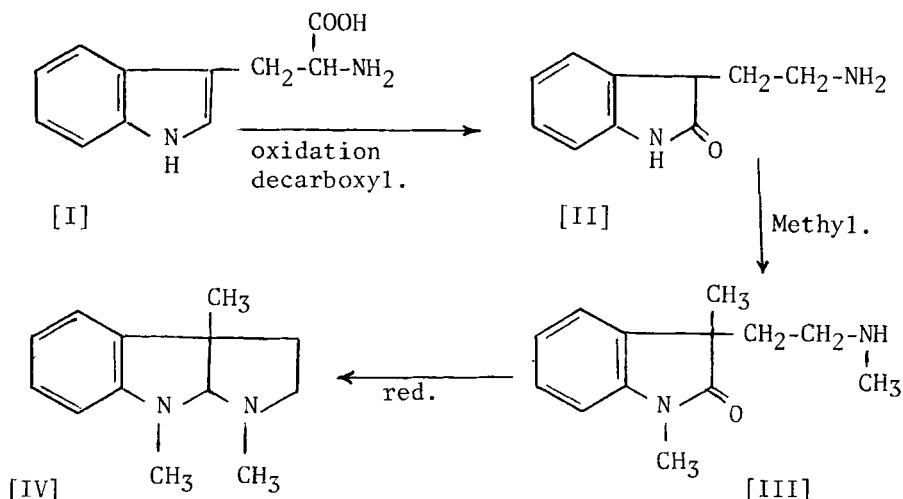
*Scheme V : The Fifth Total Synthesis*

R = H Desoxyeseroline

R = OEt Eserethole

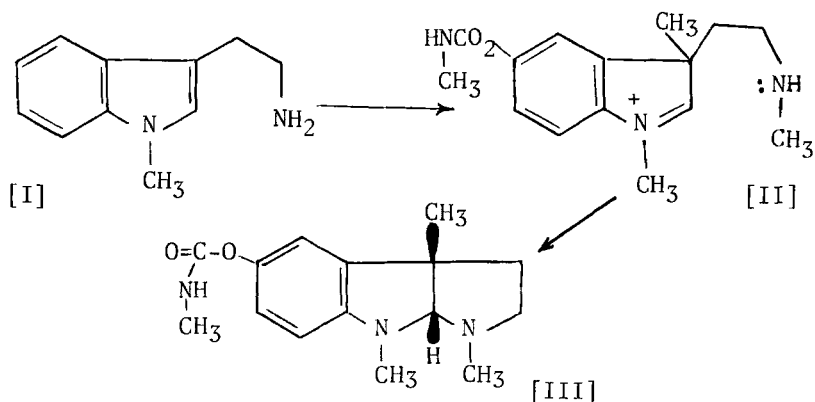
# 5. Biosynthesis of Physostigmine

Postulation of the biosynthetic pathway of physostigmine started in 1925 with the suggestion of Robinson to Barger et al ( 2,3) that this alkaloid might be built up by the plant from oxytryptophan in a manner indicated by the formulas [I]  $\longrightarrow$  [IV].



Conversion of [II] to [III] and the reduction of [III] into [IV] have been realized in practice in the first total synthesis of physostigmine (3) (Scheme I: [IV]  $\longrightarrow$  [VI]).

Later, it has been proposed that physostigmine is built up in plants from N-methyltryptamine according to the following formulas ( 31 ).





## 6. Pharmacokinetics and Metabolism

### 6.1 Absorption

Physostigmine is readily absorbed from the gastrointestinal tract, mucous membranes and subcutaneous tissues (16,32,33,34).

Following oral administration of 4 mg physostigmine salicylate (2.68 mg physostigmine base) in a healthy subject, peak serum concentrations occurred in 45 minutes (approximately 0.9 ng/ml), falling to undetectable serum levels at 3 hours (35).

Absorption of physostigmine salicylate following oral administration shows considerable interindividual variation. In one study in several healthy individuals who received 2 or 3 mg of physostigmine salicylate orally as an aqueous, 5-12% of the dose reached systemic circulation unchanged (34). Peak blood or plasma concentrations of the drug occurred within 20-30 minutes following oral administration as a solution or tablet in healthy individuals (34). Following oral administration of a single 2 mg dose of the salicylate salt in several healthy individuals, peak blood or plasma concentrations were 0.45-2.3 ng/ml (34). In one study, a volunteer received 0.5 mg subcutaneously, peak blood concentration of 1.72 ng/ml was reached at 20 minutes (35).

### 6.2 Distribution

Physostigmine is widely distributed throughout the body. It possesses the ability to cross the blood-brain barrier (as it is a tertiary amine). This is in contrast to neostigmine and pyridostigmine (36).

### 6.3 Metabolism

Physostigmine is rapidly hydrolysed at the ester linkage by cholinesterase (33,37).

### 6.4 Excretion

Although the excretion of physostigmine is not completely understood, it is known that very small amounts of it are excreted in the urine (34).

### 6.5 Half-life

Physostigmine is relatively short acting, possessing a half-life of 1 to 2 hours ( 38 ). Other study showed that in several healthy individuals, a terminal elimination half-life of 15-40 minutes has been reported (34).

### 6.6 Onset

Maximum onset of action occurs within five minutes after IV injection (39). Following topical application of a 0.25-1.0% solution or ointment of physostigmine salts to the conjunctival sac, miosis occurs within 10 to 30 minutes and persists 12 to 48 hours (34).

### 6.7 Duration of Action

Duration may be less than 1 hour requiring repeated doses at 30 to 60 minute intervals ( 40 ) or as long as 3 to 4 hour intervals (41).

The duration of action of physostigmine as an anticholinergic antidote is 0.5 to 4 hours (42).

When used as a miotic drug in the treatment of glaucoma, it has a duration of action of 12 to 36 hours (43).

## 7. Drug Stability

Physostigmine and salts acquire a red tint on contact with metals or after long exposure to heat, light and air ( 34 ). They should be kept in well closed containers protected from light (14).

In aqueous solutions, particularly in the presence of an alkali, physostigmine and its salts undergo a progressive change in color, at first becoming red, then blue and finally brown ( 33). This decomposition of physostigmine involves first hydrolytic cleavage of its methyl carbamyl group with loss of methylamine and carbon dioxide and formation of the hydroxy derivative *eseroline*; this compound is then oxidized to a dioxy derivative *rubreserine*, so named because of its red color; further degradation results in the formation of the colored derivatives *eserine blue* and *eserine brown*. Both rubreserine and eserine blue inhibit destruction of acetylcholine by cholinesterase, but both are considerably weaker than physostigmine ( 44 ). Eseroline exerted practically no effect in inhibiting cholinesterase ( 33). This studies indicate that coloration of physostigmine solutions always evidences some loss of potency and eventually the solution become therapeutically worthless ( 33). The decomposition may be retarded by previously rinsing out the bottle with diluted hydrochloric acid, and subsequently with distilled water, or by coating the inner surface of the dispensing vial with paraffin (33). Preservation of physostigmine solutions for 6 months by addition of 1:1000 of ascorbic acid has been claimed (45 ). Potassium metabisulfite or sodium bisulfite, in concentrations of 0.1% have been also reported as satisfactory preservatives for physostigmine salicylate solutions (34).

*The solution of physostigmine salicylate should preferably be freshly prepared.*

This solution should not be sterilized by heat and should be stored in tight, light-resistant containers at a temperature less than 40°C, preferably between 15-30°C; freezing should be avoided ( 34 ). Commercially available physostigmine salicylate injection has an expiration date of 2 years following the date of manufacture (34).

## 8. Methods of Analysis

### 8.1 Identification

The following identification tests have been mentioned under physostigmine salicylate in the B.P. of 1980 (14).

- To a 5 ml of a 1% w/v solution add about 0.15 ml of M sodium hydroxide; a precipitate is formed which dissolves in excess M sodium hydroxide giving a red color.
- Heat in a porcelain dish 10 mg of physostigmine salicylate with about 0.15 ml of 6 M ammonia; an orange solution is produced. The residue obtained on evaporation is soluble in ethanol (96%) giving a blue solution. Add about 0.15 ml of glacial acetic acid; the color changes to violet and on dilution with water exhibits an intense red fluorescence.
- Add to a 1% w/v solution, a 1.3% w/v solution of iron (III) chloride hexahydrate; a violet color is obtained which persists on the addition of 2 M acetic acid or ethanol (96%).
- To 15 ml of 1% w/v solution, add about 0.15 ml of hydrochloric acid; melting point of the precipitate, after washing and drying, about 158°C.

Other tests for the identification of physostigmine have been reported.

- When physostigmine is heated in potassium hydroxide solution, a deep yellow color is produced. It is stated that this color reaction can detect as little as 10 µg of the alkaloid. Under controlled conditions, the intensity of this color may be measured spectrophotometrically at 470 nm and serves as a quantitative determination (46).
- Physostigmine with a solution of phosphomolybdic acid and ammonium metavanadate in sulfuric acid, produces an emerald green color (46).
- When a solution of ammonium vanadate is added to physostigmine; a yellow brown color is produced (sensitivity 1.0 µg) (16).
- When a small quantity of physostigmine is heated in a porcelain dish with one or two drops of fuming nitric acid; a yellow color is produced, upon evaporation to dryness forms a green residue, which after cooling dissolves in alcohol to give purple color (16,46).
- Physostigmine gives a characteristic fluorescence when it is subjected to radiation of a Hg lamp filtered through a NiO glass (47).

## 8.2 Microcrystal Formation

The microcrystals of physostigmine were performed on a solution of physostigmine salicylate in water (1 mg in 1 ml). 1 to 2 drops of this solution were treated with an equal quantity of the specific reagent on a microscopical slide. After a specific time, the crystals so formed were microscopically examined (48). Shapes of the crystals are presented in table 7.

Table 7 Microcrystals of Physostigmine

Plate	Reagent (16)	Time of formation minute	Shapes of Crystals
1	Picric acid	5	Radiating irregular plates.
2	Lead iodide	2-3	Serrated blades (16)
3	Gold chloride	4	Radiating small rods.
4	Mayer's	3-4	Small rectangular plates.
5	Wagner's	1-2	Sharp radiating needles.
6	Potassium permanganate	immediate	Long solitary needles.

## 8.3 Titrimetric Methods

### 8.3.1 Non-aqueous Titration

A non aqueous titration procedure for the determination of physostigmine salicylate was described in the E.P. 1973 (49). Dissolve about 0.25 g accurately weighed physostigmine salicylate in 25 ml  $\text{CHCl}_3$ , add 25 ml glacial acetic acid and titrate with 0.02N perchloric acid in dioxane. Each 1 ml of 0.02N perchloric acid = 0.00827 g of physostigmine salicylate.

The B.P. 1980 procedure (14) make use of 0.2 g physostigmine salicylate in 15 ml of a mixture of equal volumes of  $\text{CHCl}_3$  and anhydrous glacial acetic acid to be titrated in non aqueous media with 0.1 M perchloric acid. The

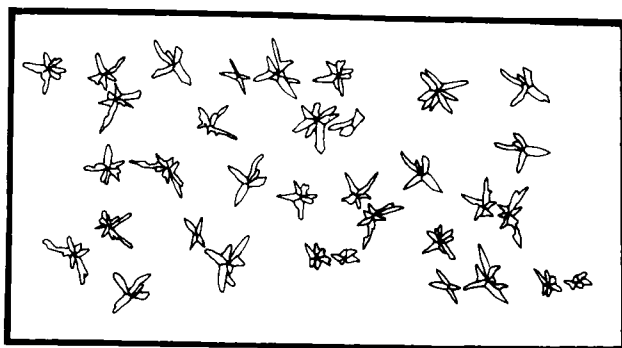


PLATE 1 : MICROCRYSTALS OF PHYSOSTIGMINE  
WITH PICRIC ACID.

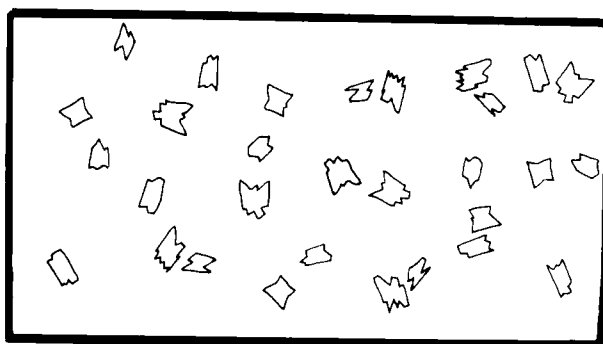


PLATE 2 : MICROCRYSTALS OF PHYSOSTIGMINE  
WITH LEAD IODIDE.

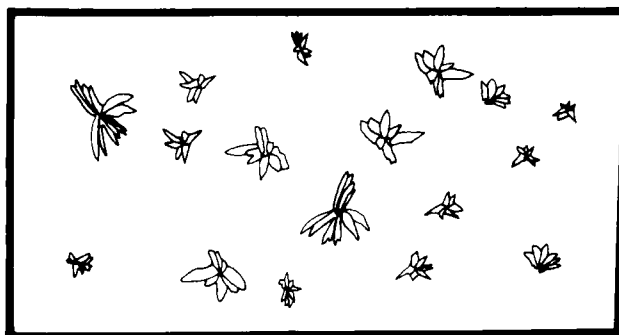


PLATE 3 : MICROCRYSTALS OF PHYSOSTIGMINE  
WITH GOLD CHLORIDE.

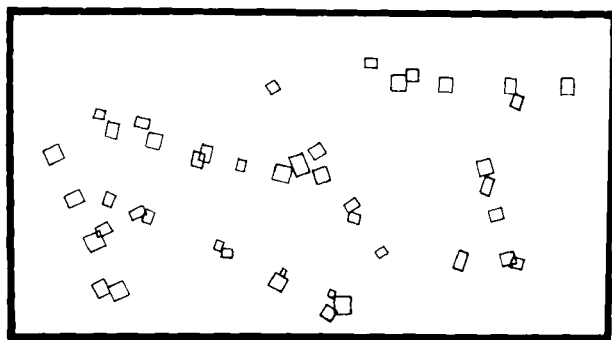


PLATE 4 : MICROCRYSTALS OF PHYSOSTIGMINE  
WITH MAYER'S REAGENT.

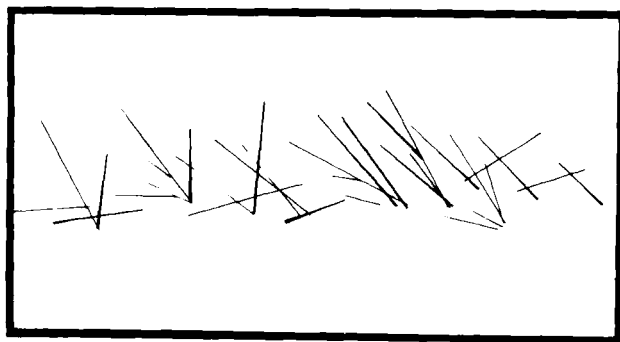


PLATE 5 : MICROCRYSTALS OF PHYSOSTIGMINE  
WITH WAGNER'S REAGENT.

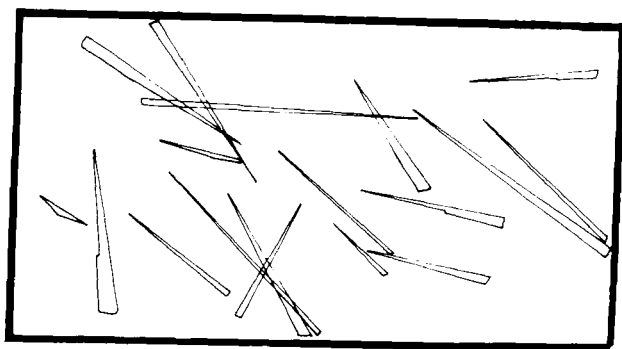


PLATE 6 : MICROCRYSTALS OF PHYSOSTIGMINE  
WITH POT. PERMANGANATE.

end point is determined potentiometrically. Each 1 ml of 0.1 M perchloric acid  $\equiv$  0.04135 g of physostigmine salicylate.

The USP XX ( 50 ) described an assay procedure for physostigmine and physostigmine salicylate using 175 mg and 250 mg respectively. The weighed amount, dissolved in 25 ml  $\text{CHCl}_3$ , 25 ml glacial acetic acid added and the solution was titrated with 0.02 N perchloric acid in dioxane VS. The end point was determined potentiometrically. Each 1 ml of 0.02 N perchloric acid is equivalent to 5.507 mg physostigmine and 8.270 mg physostigmine salicylate. For physostigmine sulfate, 200 mg in 25 ml of water are to be used. The solution rendered alkaline by the addition of about 1 g of sodium bicarbonate and extracted with one 25 ml and five 10-ml portions of  $\text{CHCl}_3$ , each time shaking vigorously for 1 min. To the filtered combined extract, 15 ml of glacial acetic acid and 10 ml of acetic anhydride are added and the amount of physostigmine sulfate determined by non aqueous titration with 0.02 N perchloric acid as above. Each ml of 0.02 N perchloric acid is equivalent to 6.488 mg of physostigmine sulfate. Solution of 0.03-0.1% physostigmine salicylate was analyzed by adjusting a sample of injection solution to pH 8-9 with  $\text{NaHCO}_3$ , extracting 3 times with  $\text{CHCl}_3$ , filtering the extracts and titrating in the presence of dimethyl yellow by 0.005 N p-toluenesulfonic acid in dioxane ( 51 ).

Another method using arylsulfonic acids in quantitative analysis of alkaloids in non aqueous media was reported ( 52 ). As titrants were used 0.005 N dioxane solutions of the following acids : naphthalene-sulfonic acid, naphthalene-2-sulfonic acid, 5-nitronaphthalene sulfonic (I), 2-naphthol-6-sulfonic (II), 2-methoxynaphthalene 6-sulfonic and 2-propoxynaphthalene-6-sulfonic (III). The titrants contained 1% phenol and were standardized against atropine or brucine dissolved in  $\text{CHCl}_3$  using 0.1% dimethyl yellow as indicator. For the determination, 5 ml of a solution to be analyzed were taken containing



~ 5 mg alkaloid salt, its pH adjusted to 8-9 with saturated  $\text{NaHCO}_3$  solution or 5%  $\text{NaOH}$ , and the solution was extracted 4-5 times with 5-10 ml  $\text{CHCl}_3$  each. The combined extracts were filtered and titrated. Many alkaloids including physostigmine were thus determined with the error  $\leq \pm 1\%$ . Best results were obtained with I-III as titrants.

#### 8.3.2 Potentiometric Titration

A potentiometric titration method using 2.5% Na tetraphenyl borate and valinomycin ion selective electrode for determination of physostigmine salicylate was reported (53).

#### 8.3.3. Iodometric Determination

A method was described for iodometric determination of physostigmine salicylate. (10-20 hr. reaction at room temperature in  $\text{H}_2\text{O}$ -HOAC and  $\text{H}_2\text{O}$ -NaCl mineral acid systems) (54).

### 8.4 Spectrophotometric Methods

#### 8.4.1 Colorimetric Determination

A method based upon the colorimetric determination for rubreserine formed by hydrolysis and oxidation of physostigmine in aqueous solution was reported (55). This method was employed by another author (56) who reported that under the same conditions a similar solution containing ascorbic acid 0.1% in place of sodium metabisulfite lost 25% of its content of physostigmine. Investigations of the effects of pH and temperature on the stability of physostigmine have been facilitated by the development of specific analytical methods for the determination of the drug in the presence of its degradation products. Fletcher and Davies (57) and Smith (58) have used modifications of the method of Haugas and Mitchell (59), based upon the reaction of physostigmine with nitrous acid to form a yellow compound that can be extracted with  $\text{CHCl}_3$  and determined colorimetrically. In a study of stability

of physostigmine eye drops BPC, Rogers ( 60) proposed the following method for its colorimetric determination.

To 10 ml of a 10% v/v solution of lactic acid in a 100 ml separator was added 0.2 ml of the test solution of physostigmine sulfate and 1 ml of a 1% w/v solution of sodium nitrite, the solutions were mixed by swirling. After 30 min, 1 g of ammonium sulfonate was added, and the yellow compound was extracted with 10, 5 and 5 ml portions of  $\text{CHCl}_3$ ; the combined  $\text{CHCl}_3$  extracts were diluted to 25 ml with  $\text{CHCl}_3$ . The absorbance of the  $\text{CHCl}_3$  solution was measured at 417 nm, with  $\text{CHCl}_3$  as a blank, in an SP 500 spectrophotometer with 1 cm cells. The coefficient of variation in the assay of ten 0.2 ml samples of a freshly prepared 0.5% w/v solution of physostigmine sulfate was 0.98%.

Other colorimetric procedures were also described (46,61).

#### 8.4.2 UV Determination

Determination of physostigmine by UV spectrophotometry after separation of the intact drug from its degradation products by extraction with cyclohexane - amyl alcohol (4:1) was reported (62).

Another method involving separation of physostigmine from its degradation products by TLC on alumina, with  $\text{CHCl}_3$  - acetone (5:4) as the solvent, was presented ( 63). Physostigmine was eluted with methanolic HCl and determined by UV spectrophotometry. Two methods were used for the correction of irrelevant absorbance. A differential method in which absorbance measurements were made at three wavelengths, and a method in which orthological functions were applied to absorbance measurements at a set of nine wavelengths. The reproducibility of the elution method appears to be slightly better than that of the direct reflectance method (coefficient of variation, 5.81%) but the technique of elution is laborious and slow. Both methods give more reproducible results than the gas chromatographic method of Teare and Borst for physostigmine salicylate (coefficient of variation,

11.5%). Analysis of physostigmine and pilocarpine in the same solution has been studied (56).

The alkaloids have not been separated but have been determined by two different spectrophotometric methods.

The USP XX ( 50 ) reported two spectrophotometric methods for assay of physostigmine salicylate in ophthalmic solution and physostigmine sulfate in ophthalmic ointment as follows:

A- For physostigmine salicylate ophthalmic solution:

Dissolve a suitable quantity of USP physostigmine salicylate R.S. previously dried and accurately weighed, in water to obtain a standard solution having a known concentration of about 5 mg/ml. Transfer 2.0 ml of the standard solution to a separator, and transfer an accurately measured volume of physostigmine salicylate ophthalmic solution, equivalent to about 10 mg of physostigmine salicylate, to another separator, and treat each solution as follows:

Add 10 ml of pH 7.8 phosphate buffer and extract successively with one 30-ml and three 20-ml portions of ether, collecting the combined extracts in a separator. Extract the combined ether solutions with three 20-ml portions of dilute HCl (1 in 1000), combine the acid extracts in a 100-ml volumetric flask, add dilute HCl (1 in 1000) to volume and mix. Dilute 20.0 ml of this solution with dilute HCl to 100 ml, and mix. Determine the absorbances of both solutions in 1-cm cells at the wavelength of maximum absorbance at about 246 nm with a suitable spectrophotometer, using dilute HCl as the blank. Calculate the quantity, in mg. of physostigmine salicylate in the portion of ophthalmic solution taken by the formula  $2C (Au/As)$ , in which C is the concentration, in mg/ml, of USP physostigmine salicylate R.S. in the standard solution, and Au and As are the absorbances of the solution from the ophthalmic solution and the standard solution, respectively.

B- For physostigmine sulfate ophthalmic ointment:

Standard preparation - Transfer about 12 mg of USP physostigmine sulfate R.S., accurately weighed, to a 200-ml volumetric flask. Add water to volume, and mix.

Assay preparation - Transfer to a separator a weighed quantity of physostigmine sulfate ophthalmic ointment, equivalent to about 12 mg of physostigmine sulfate. Add 100 ml of ether, and shake until the ointment base dissolves. Extract with four 30-ml portions of water, collect the aqueous extracts in a 200-ml volumetric flask, add water to volume and mix.

Procedure - Concomitantly determine the absorbances of both solutions in 1-cm cells at the wavelength of maximum absorbance at about 305 nm, with a suitable spectrophotometer, using water as the blank. Calculate the quantity, in mg of physostigmine sulfate in the portion of the ointment taken by the formula  $200C (Au/As)$ , in which C is the concentration, in mg/ml, of USP physostigmine sulfate RS in the standard preparation, and Au and As are the absorbances of the Assay preparation and the standard preparation, respectively.

8.4.3 Spectrofluorimetric

A procedure for spectrofluorimetric determination of physostigmine was reported (64).

8.4.4 Phosphorescent Analysis

A phosphorescent principal was adopted for the quantitative determination of physostigmine (65).

8.5 Enzymatic Methods

A quantitative enzymatic assay for measuring physostigmine in human whole blood was described (66) The authors stated that since the drug is given to patients in low doses (2 mg) and its metabolism in the body is quite rapid, only nanogram levels of the

drug are found in the blood and existing colorimetric, spectrofluorometric, phosphorescent and TLC methods are not sufficiently sensitive to measure these low levels. Blood samples maintained at  $37^{\circ}$ , and cholinesterase activity was measured periodically. The time required for enzyme reactivation is related to physostigmine concentration. The precision of the method was within  $\pm 4\%$  over a physostigmine concentration range of  $0.5\text{--}50 \times 10^{-7}\text{M}$ . The assay was carried out as follows:

A- In Vitro Assay - Microliter volumes of physostigmine solution were added, using micropipets, to 5-ml aliquots of whole blood concentration of inhibitor were 0.25, 0.50, 1.00, 1.25, 2.50, and  $5.00 \times 10^{-7}\text{M}$ ; 10 whole blood samples were prepared at each concentration. Each tube was immediately stoppered, shaken, and placed in a water bath at  $37^{\circ}$ . Zero time was recorded for each tube. At selected times, the blood samples were gently mixed; transfer pipets were used to remove aliquots to sample cups for enzyme assay. Each aliquots was assayed within 1min. after removal from the incubation bath.

B- In Vivo Assay - Physostigmine was administered intravenously to 10 subjects and intramuscularly to nine subjects. Blood samples for the physostigmine assay were collected from all subjects 30 min. after dosing. These samples were placed immediately in a  $37^{\circ}$  incubation bath, and the time recorded. Cholinesterase activity was measured periodically as described. The concentration obtained is a measure of the physostigmine present in the blood at the time the sample is withdrawn.

#### Cholinesterase measurement:

Whole blood cholinesterase activity was determined by an automated procedure (67). Enzyme activity was expressed as micromoles of substrate hydrolyzed by 1 ml of sample per min. of incubation time. The substrate was acetylthiocholine iodide at  $2 \times 10^{-3}\text{M}$ . The reaction mixture, containing sample, substrate, and 5, 5'-dithiobis (2-nitro-benzoic acid (I) was buffered to pH 8.2 with 0.05 M tromethamine and incubated for approximately 6 min. at  $37^{\circ}$ . Thiocholine, resulting from enzymatic hydrolysis of the substrates reacts with I to form a colored anion. This colored anion is dialyzed into pH 8.2 buffer, and the absorbance was measured at 420 nm.

## 8.6 Chromatographic Methods

### 8.6.1 Paper Chromatography

Clarke (16) described the following technique which was reported for the analysis of nitrogenous bases including physostigmine. Whatman No.1 sheets were buffered by dipping in a 5% solution of sodium dihydrogen citrate and a solvent composed of 4.8 g citric acid in a mixture of 130 ml water and 870 ml of n-butanol was used. An  $R_f$  value of 0.43 was reported for physostigmine (68,69).

The following systems were also employed for the identification of physostigmine.

<u>Solvent system</u>	<u>Paper</u>	<u>Ref.</u>
Pet. ether (saturated with formamide)-benzene-ethanol (100:25:25)	impregnated with formamide + 8% ammonium formate	(70)
Butanol-HCl (conc.)-water (50:75:17.5)		
Butanol-acetic acid-water (4:1:5)		
Chloroform	impregnated with formamide	(71)
Chloroform mixture with benzene, toluene-xylene		

### 8.6.2 TLC (Thin Layer Chromatography)

A TLC method for separation of physostigmine ( $R_f = 0.74$ ) from its degradation products, eseroline ( $R_f = 0.51$ ) and rubreserine ( $R_f = 0.55$ ) on silica gel with  $\text{CHCl}_3$ -acetone-33% w/w dimethylamine in ethanol (5:4:1) as the solvent was reported (72).

The physostigmine was eluted with 0.1N NaOH and the rubreserine formed by hydrolysis and oxidation was determined colorimetrically at 480 nm. The same author attempted to elute the alkaloid with various organic solvents but the recoveries were low. The previous method, however, was excised by Smith (73) who reported that rubreserine reacted with dimethylamine for 10 min. to form a yellow product with an  $R_f$  (0.73) close to that of physostigmine. Physostigmine and its degradation products after 5 years storage were examined by TLC and direct-reflectance spectrophotometry (74).

Identification of some alkaloids, including physostigmine, in chemical toxicological analysis using TLC silica gel was discussed (75).

Alkaloids were extracted from biological material with 0.02 N  $\text{H}_2\text{SO}_4$  at pH 2.5-3.0. The extract was alkalinized with 20% NaOH to pH 8-9, extracted with  $\text{CHCl}_3$  and chromatographed on TLC of silica gel KSK.  $\text{CHCl}_3$  -  $\text{Me}_2\text{CO}$ - $\text{Et}_2\text{HN}$  (50:30:2);  $\text{Et}_2\text{O}$  -  $\text{Me}_2\text{CO}$  -  $\text{NH}_3$  (40:20:2) and  $\text{CHCl}_3$  -  $\text{Me}_2\text{CO}$ - $\text{NH}_3$  (30:30:2) were the solvent systems. The  $R_f$  of physostigmine in three different solvent systems was reported (76): 0.55 in  $\text{MeOH}$  - strong ammonia (100:1.5), 0.12 in Cyclohexane - toluene - diethylamin (75:15:10) and 0.36 in  $\text{CHCl}_3$  -  $\text{MeOH}$  (90:10) acidified potassium permanganate solution was used for location of the spots.

#### 8.6.3 HPLC(High Performance Liquid Chromatography)

An HPLC method was developed for determination of pilocarpine, physostigmine and its degradation product rubreserine and preservatives (77). The column (30 cm X 3.9 mm) was packed with U Bondapak  $\text{C}_{18}$  (pore size 10  $\mu\text{m}$ ). The mobile phase was 40% methanol with 0.005 M heptane sulfonic acid, pH 3.6, filtered through 0.5  $\mu\text{m}$  Celotat<sup>(R)</sup>, flow rates 1ml/min. Samples of 80  $\mu\text{g}$  pilocarpine and 8  $\mu\text{g}$  physostigmine were injected. A wave length at 235 nm was found suitable for the detection of pilocarpine, physostigmine and preservatives. For the detection of the degradation product rubreserine, a wave length of 292 was chosen. As internal standard for unpreserved preparations, methyl p-hydroxybenzoate can be used. If the eye-drops are preserved with methyl p-hydroxybenzoate for instance, ethyl p-hydroxybenzoate can be used. Fig.10 shows the separation of pilocarpine, physostigmine, methyl p-hydroxybenzoate and propyl p-hydroxybenzoate. Physostigmine salicylate forms two ion pairs and also two peaks, one due to salicylate after 4 min. and one due to physostigmine after about 13 min. Methyl p-hydroxybenzoate appears after 11 min. After about 15 min., the flow is increased to 2 ml/min. in order to accelerate the elution of propyl p-hydroxybenzoate. Another (Fig.11) shows a chromatogram of an aged physostigmine solution (red

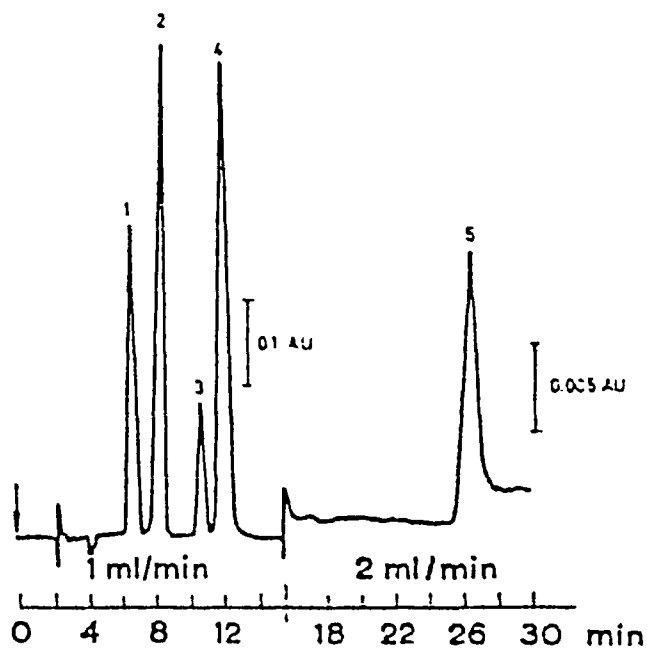


Fig. 10 HPLC of pilocarpine, physostigmine and preservatives. Peaks: 1 = pilocarpine; 2 = salicylate; 3 = methyl *p*-hydroxybenzoate; 4 = physostigmine; 5 = propyl *p*-hydroxybenzoate. Detection: UV, 235 nm. (77).



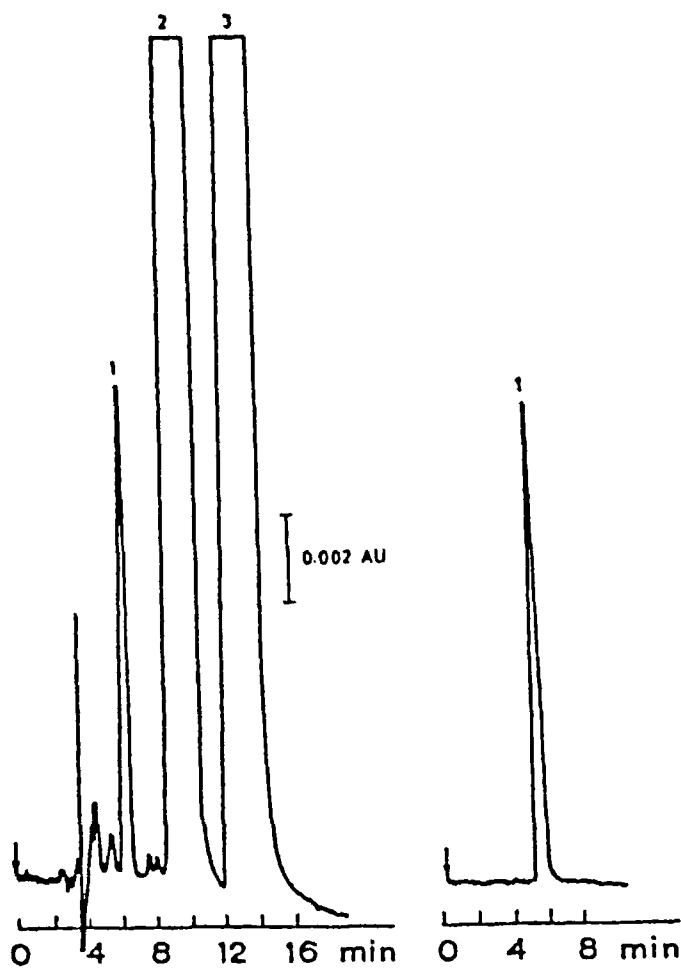


Fig.11 HPLC of an aged physostigmine solution (left) and a rubreserine standard solution (right).  
Peaks: 1 = Rubreserine; 2 = salicylate; 3 = physostigmine. Detection: UV, 292 nm. (77).

due to the degradation of physostigmine). The capacity factors, sample concentrations and detection limits for physostigmine, rubreserine and pilocarpine have been calculated. The relative standard deviation calculated for the peak height was 0.3% for physostigmine. The author stated that the described method is rapid and accurate where by a separation and quantitative analysis of rubreserine, pilocarpine, methyl p-hydroxybenzoate and physostigmine is completed in Ca 15 min.

Quantitative HPLC determination of the cholinesterase inhibitor physostigmine in brain tissue using reversed phase HPLC was discussed (78) Liprosorb RP-8 column and MeOH-H<sub>2</sub>O-NaH<sub>2</sub>PO<sub>4</sub>

sodium perchlorate mobile phase and UV at 254 nm for detection.

Another HPLC procedure (79) was developed to measure plasma concentrations after a single subcutaneous injection of 1 mg physostigmine salicylate (equivalent to 0.67 mg of the base). The author stated that the existing enzymatic method (66) with a sensitivity of Ca 0.7 ng ml<sup>-1</sup> in blood was considered unsuitable.

A stainless-steel column, 250X4.5 mm I-D, was slurry packed with 5-um silica particles in methanol. The eluent was methanol - 1:1 ammonium nitrate buffer, pH 8.6 (9:1) and degassed to remove dissolved oxygen before use. The flow-rate was maintained at 1ml/min. Detection was by either a fixed-wave length (254 nm) UV detector or bioanalytical systems electro-chemical detector. The method was carried out as follows:

Plasma samples: A male volunteer, aged 34 years and weighing 72 kg, was injected subcutaneously with 1 ml physostigmine salicylate solution B.P. (equivalent to 0.67 mg base). Venous blood (10 ml) was withdrawn into heparinised tubes and neostigmine bromide solution (1 mg ml<sup>-1</sup>, 10 ul) added. The blood was mixed and centrifuged at 4° to separate the plasma, after which it was stored at 4° until assay later in the day. Samples were taken before and at 15,30,60,90 and 120 min after injection.

Extraction procedure - Plasma (3 ml) and ammonium hydroxide solution (1M, 1ml) were pipetted into a screw-cap tube. Diethyl ether (5 ml) was added and the capped tube shaken mechanically for 20 min. After centrifugation the ether layer (4 ml) was transferred to a pointed tube and evaporated at 40° under a gentle stream of nitrogen. The residue was dissolved in methanol (60  $\mu$ l) and 50  $\mu$ l injected into the chromatograph.

Standard solutions were prepared at 20, 10, 5, 2, 1, 0.5 and 0 ng ml<sup>-1</sup> in plasma containing neostigmine bromide (10  $\mu$ g ml<sup>-1</sup>) and taken through the extraction procedure along with the unknown samples.

A fourth HPLC method (35) has been developed for the analysis of physostigmine salicylate in blood and urine samples after oral doses. A healthy 38-years old female volunteer (5g/kg) was given physostigmine solution to drink on three separate occasions, the doses progressively increasing from 1 to 2 to 4 mg of the salicylate salt (0.67, 1.34, and 2.68 mg physostigmine base). The dose was taken with 100ml water. Blood samples were taken via an indwelling cannula and urine was collected every 30 min. or so. After the two lower doses blood physostigmine concentrations were below the limit of detection (50 pg/ml). However, low concentrations were measurable in urine. After the 2.68 mg dose, the physostigmine concentration was greater in the 45 min. blood sample concentration then fell, with an apparent first-order half time of 25-30 min. to become undetectable by 3 h. The maximum urinary excretion rate (1-2  $\mu$ g/h) was measured in urine collected between 25 and 50 min.

Low doses (typically 0.5-2 mg) of physostigmine, coupled with rapid metabolism mean that a plasma assay must be capable of measuring nanogram or even sub-nanogram amounts.

Unmodified silica columns together with non-aqueous ionic eluents were reported for the analysis of basic drugs by HPLC (80). Retention and relative response data (UV, 254 nm and electrochemical, +1.2 V) have been

generated for 462 compounds, including physostigmine, using a 125-mm Spherisorb S5W silica column and methanolic ammonium perchlorate (10 mM, pH 6.7) as eluent. This system can be used isocratically in qualitative analyses and also for quantitative work, when either the wavelength or the applied potential can be adjusted to optimise the response.

#### 8.6.4 GLC (Gas Liquid Chromatography)

Very few works dealing with the gas chromatographic analysis of physostigmine, was traced in the current literature. The alkaloid showed relative retention time (related to codeine) 0.2 and 0.29 (Clarke (16) when analyzed under the following conditions: A) column, 2.5% SE30 on 80-100 mesh Chromosorb W, 5 ft X 4 mm i.d., temperature of analysis, 225°C, carrier gas, N<sub>2</sub> with flow rate of 50 ml/min; detector, FID, H<sub>2</sub>, 50 ml/min and air 300 ml/min. and B) column 3% XE-60 silicone nitrile polymer on 100-120 mesh Chromosorb W with the same conditions of A. Other conditions including the use of a glass column 2 m X 4 mm i.d. packed with 25% SE-30 on 80-100 mesh Chromosorb G (acid-washed and dimethyldichlorosilane-treated) and carrier gas flow of 45 ml/min were also reported for analysis of physostigmine (81).

The following conditions were established in our laboratory for the GLC analysis of physostigmine:

Column : 3% OV-1 on Gas Chrom Q 80/100 mesh;  
Temp. 250°C increased to 300°C by the rate of 10°/min; detector. FID at 250°C, Carrier gas, N<sub>2</sub> with a flow rate of 40 ml/min, gases for the detector, H<sub>2</sub> with 30 ml/min. and air with 300 ml/min; injector temp., 250°C.

Under these conditions physostigmine base showed a retention time 1.3 min. For the salicylate, few drops of ammonia were added to the chloroformic solution before analysis.

## 8.7 Other Methods

### 8.7.1 Polarographic Methods

Oscillographic polarography has been employed to determine the stability of physostigmine ophthalmic solutions. Such solutions were found to be most stable at pH value between 2.0 and 4.5 (82,83).

### 8.7.2 Coulometry

Physostigmine sulfate was determined by a coulometric method, a precision of 1.0 CV was reported (84).

## 9. Therapeutic Uses

Physostigmine is long well known as an anticholinesterase inhibitor i.e. it is a *cholinergic drug*.

As physostigmine is a tertiary amine alkaloid, it has the ability to penetrate the blood brain barrier, while neostigmine (a synthetic related derivative) which is a quaternary ammonium ion, is not capable of crossing the barrier (37, 85).

Physostigmine is a reversible anticholinesterase which effectively increases the concentration of acetylcholine at the sites of cholinergic transmission, it therefore inhibits the destruction of acetylcholinesterase and thereby prolongs and exaggerates the effect of acetylcholine (85). It can reverse both central and peripheral anticholinergia.

It is therefore used to reverse the effect upon the central nervous system, caused by clinical or toxic dosages of drugs capable of producing the anticholinergic syndrome (85). Physostigmine can reverse such syndrome as delirium and hallucination resulting from belladonna alkaloid overdose (86,87); acute tricyclic antidepressant poisoning (88); intoxication from atropa powders (scopolamine) and sleeping preparations (89,90); phenothiazine and diazepam-induced coma (91); antihistamine-induced excitement and depression (37) and reversal of amitriptyline intoxication (41).

Since physostigmine is rapidly hydrolyzed by cholinesterase (92), repeated therapeutic doses may be necessary at 30 to 60 minute intervals (37).

Ocular therapy

Anticholinesterase agents particularly physostigmine (as the salicylate salt) are of great value in the management of the primary type glaucoma as well as certain categories of the secondary type (93).

Physostigmine produces a fall in intraocular pressure in primary type glaucoma (93).

Low concentrations of physostigmine are reported to decrease the blurred vision and pain associated with this condition (94).

In alternation with a mydriatic drug such as atropine, physostigmine has proven useful for the breaking of adhesions between the iris and the lens or cornea (93,95,96).

Alzheimer's disease

A deficiency of functional cholinergic neurons, has been observed by several groups in patients with progressive dementia of Alzheimer type. Physostigmine has been employed in the earlier stages of the disease to improve memory. Results have been variable, although some investigators have employed dosage schedules that appear to cause transient improvement (97).

In Myasthenia gravis

Physostigmine has been used in Myasthenia gravis (98), but for this purpose it is now largely replaced by neostigmine (93).

Other articles and reviews dealing with the pharmacology and therapeutic uses of physostigmine have been reported (99-103).

ACKNOWLEDGEMENT

The authors would like to thank Mr. Uday C. Sharma, Department of Pharmacognosy, College of Pharmacy for his valuable help in typing the manuscript.

## REFERENCES

1. T.E. Wallis, "Textbook of Pharmacognosy", 5th ed., p.201, J. and A. Churchill Ltd., London (1967).
2. E. Stedman and G. Barger, J. Chem. Soc., 247 (1925).
3. P.L. Julian and J. Pikel, J. Am. Chem. Soc., 57, 755 (1935).
4. J. Harley-Mason and A.H. Jackson, J. Chem. Soc., 3651 (1954).
5. P. Rasenmund and A. Sotiriou, Angew. Chem., 76, 787 (1964).
6. M. Ikeda, S. Matsugashita and Y. Tamura, J. Chem. Soc. (Perkin Trans. 1), 1770 (1977).
7. J.B. Winjberg and W.N. Speckamp, Tetrahedron, 34, 2399 (1978).
8. J.C. Grasselli and W.M. Ritchey "Atlas of Spectral Data and Physical Constants for Organic Compounds", 2nd ed., Vol. IV p. 158, CRC Press Inc., Cleveland, Ohio (1975).
9. R.B. Longmore and B. Robinson, Chem. & Ind. (London) 622 (1969).
10. R.K. Hill and G.R. Newkome, Tetrahedron, 25, 1249 (1969); G.R. Newkome and N.S. Bhacca, Chem. Commun., 385 (1969).
11. T.J. Petcher and P. Pauling, Nature, 241, 277 (1973).
12. "The Merck Index", 10th ed., M. Windholz Edit., Merck and Co. Inc., Rahway, N.J. (1983).
13. "The United States Dispensatory" 27th ed., A. Osol, R. Pratt and A. Gennaro Edits., p. 912, J.B. Lippincott Co., Philadelphia (1973).
14. "The British Pharmacopoeia", p. 349, Her Majesty's Stationary Office, Cambridge (1980).
15. "Specification for the Quality Control of Pharmaceutical Preparations" World Health Organization, Geneva (1967).

16. E.G.C. Clarke "Isolation and Identification of Drugs" Vol. 1, p. 498, "The Pharmaceutical Press", London (1978).
17. G.A. Cordell "Introduction to Alkaloids, A Biogenetic Approach", p. 588, John Wiley and Sons, New York (1981).
18. P.A. Crooks, B. Robinson and O. Methcohn, *Phytochem.*, 15, 1092 (1976).
19. V.I. Stenberg, N.K. Narian, S.P. Singh, R.H. Obenauf and M.J. Albright, *J. Heterocycl. Chem.*, 14, 407 (1977).
20. C. Djerassi, H.W. Brewer, C. Clarke and L.J. Durham, *J. Am. Chem. Soc.*, 84, 3210 (1962).
21. M. Ohashi, J.M. Wilson, H. Budzikiewicz, M. Shamma, W.A. Slusarchyke and C. Djerassi, *J. Am. Chem. Soc.*, 85, 2807 (1963).
22. Structure Elucidation of Natural Products By Mass Spectrometry, Vol. I : Alkaloids, H. Budzikiewicz, C. Djerassi and D.H. Williams, p. 162. Holden-Day Inc., San Francisco (1964).
23. L. Marion, A chapter in "The Alkaloids" Vol. II, R.H.F. Manske and H.L. Holmes Edits., p. 438, Academic Press, New York (1952).
24. S.C. Harvey, chapter 45 in "Remington's Pharmaceutical Sciences", 15th ed., p. 831, "Mack Publishing Co." Easton, PA (1975).
25. M. Polonovski and C. Nitzberg, *Bull. Soc. Chim. Fr.*, 19, 27 (1916).
26. H. Decker and I. Becker, *Ann.*, 395, 362 (1913).
27. A.C. Cope, C.M. Hofmann, C. Wycoff and E. Hardenburgh, *J. Am. Chem. Soc.*, 63, 5452 (1941).
28. B. Robinson, chapter 5 in "The Alkaloids, Chemistry and Physiology", Vol. IX, R.H.F. Manske Edit., p. 383. Academic Press, New York (1968).
29. E. Fischer and F. Jourdan, *Ber.*, 16, 2241 (1883); E. Fischer and O. Hess, *Ber.*, 17, 559 (1884).
30. A. Reissert, *Ber.*, 38, 1603, 3415 (1905).



31. A. ur. Rahman and A. Basha "Biosynthesis of Indole Alkaloids" p. 159, Clarendon Press, Oxford (1983).
32. Goodman and Gilman's "The Pharmacological Basis of Therapeutics", 6th ed., A.G. Gilman, L.S. Goodman and A. Gilman Edits., p. 108, Macmillan Publishing Co., Inc., New York (1980).
33. The United States Dispensatory, 27th ed., A. Osol and R. Pratt Edits., p. 912, J.B. Lippencott Co., Philadelphia (1973).
34. American Hospital Formulary Service (AHFS), Drug Information 88 p. 577, 1561, American Society of Hospital Pharmacists (1988).
35. M. Gibson, T. Moore, C.M. Smith and R. Whelpton, Lancet, 695 (1985).
36. S.P. Wright, Clin. Ped., 15, 1123 (1976).
37. J.H. Lee, H. Turndorf and P.J. Poppers, Anesthesiology, 43, 683 (1975).
38. American Medical Association (AMA), Council on Drugs, AMA Drug Evaluations, 4th ed., American Medical Association, Chicago (1980).
39. R.W. Newton, J. Am. Med. Ass., 231, 941 (1975).
40. B.H. Rumack, Pediatrics, 52, 449 (1973).
41. B.D. Snyder, L. Blonde and W.K. McWhirter, J. Am. Med. Ass., 230, 1433 (1974).
42. R.P. Granacher and R.J. Bladessarini, Arch. Gen. Psychiat., 32, 375 (1975).
43. Product Information: Antilirium (R), Forest Pharmaceuticals (1982); Isopto Eserine (R), Alcon (1982).
44. S. Ellis, J. Pharmacol. Exp. Ther., 79, 364 (1943).
45. H. Swallow, Pharm. J., 112, 11 (1951).
46. J.B. Saxton, A Chapter in "The Alkaloids", Vol. VII, R.H.F. Manske Edit., p. 146, Academic Press, New York (1960).

47. A. Andant, Bull. Sci. Pharmacol., 37, 89, 169 (1930); C.A. 24, 2053, 3441 (1930).
48. A.A. Shehata, College of Pharmacy, King Saud University at Riyadh, Private Communications (1988).
49. The Egyptian Pharmacopoeia (1973), Arabic ed., Ministry of Health, Cairo, Egypt p. 736 (1972).
50. The United States Pharmacopeia (USP XX), The National Formulary (NF XV)", p.624 "The United States Pharmacopoeial Convention Inc.", Md (1980).
51. M. Sobiczewska, Farm. Polska, 20, 35 (1964).
52. R. Stainslaw, Z. Zdzislaw and O. Barbara, Farm. Polska, 25, 263 (1969).
53. K. Vytras and V. Riha, Cesk. Farm., 26 (1), 9 (1977).
54. P.P. Suprum, Farmatsiya (Moscow), 24(5), 73 (1975); C.A. 84, 35386 s (1976).
55. H. Hellberg, Svensk Farmaceutisk Tidskrift, 53, 637, 658 (1949).
56. R. Fagerström, J. Pharm. Pharmacol., 15, 479 (1963).
57. G. Fletcher and D.J.G. Davies, J. Pharm. Pharmacol., 20, 108 s (1968).
58. G. Smith, Ph.D. Thesis, Heriot-Watt University (1972).
59. E.A. Haugas and B.W. Mitchell, through A.R. Rogers, Pharm. J., 211, 353 (1973).
60. A.R. Rogers, Pharm. J., 211, 353 (1973).
61. O.E. Schultz and R. Kratzer, Arch. Pharm., 295, 444 (1962).
62. I. Christenson, Acta Pharm. Suecica, 6, 287 (1969).
63. A.R. Rogers and G. Smith, J. Chromat., 87, 125 (1973).
64. S. Udenfriend, D.E. Duggan, B.M. Vasta and B.B. Brodie, J. Pharmacol. Exp. Ther., 120, 26 (1957).
65. J.D. Winefordner in "Fluorescence and Phosphorescence Analysis; Principles and Applications" p.169, D.M. Hercules Edit., Wiley and Sons, New York (1966).

66. W.A. Groff, R.I. Ellin and R.L. Skalsky, J. Pharm. Sci., 66, 389 (1977).
67. W.A. Groff, A. Kaminskis and R.I. Ellin, Clin. Toxicol., 9, 353 (1976).
68. A.S. Curry and H. Powell, Nature, 173, 1143 (1954).
69. E.G.C. Clarke, "Methods of Forensic Science", F. Lundquist Edit., vol. 1, p. 31, Interscience Publishers, New York (1962).
70. I.R.M. Pinyazhko, Farm. Zh. (Kiev), 17(3), 26 (1962).
71. J. Reichelt, Pharmazie, 11, 718 (1956).
72. B.H. Berg, Acta Pharm. Suecica, 3, 209 (1966).
73. G. Smith, Proceedings of the Society for Analytical Chemistry, 8, 66 (1971).
74. O.A. Akopyan, B.I. Schvydkii, S.I. Baik, D Yu. Rogovskii, Z.S. Rokach, A.I. Shkadova and O.M. Scherbina, Farm. Zh (Kiev), 4, 49 (1979).
75. A.C. Moffat "Clarke's Isolation and Identification of Drugs" Second ed. p. 900, The Pharmaceutical Press, London (1986).
76. I. Sunshine, Am. J. Clin. Path., 40, 576 (1963).
77. M. Kneczke, J. Chromat., 198, 529 (1980).
78. D.J. De Wildt, A.J. Porsius and H.H. Van Rody, J. Chromat. (Biomed. Appl.), 225, 381 (1981).
79. R. Whelpton, J. Chromat. (Biomed. appl. 23), 272, 216 (1983).
80. I. Jane and A. McKinnon, J. Chromat. (Biomed. Appl.), 323, 791 (1985).
81. R.E. Ardrey and A.C. Moffat, J. Chromat., 220, 195 (1981).
82. V. Parrák, O. Mohelska and F. Machovicova, Pharm. Zentralhalle, 100, 503 (1961).
83. V. Parrák, Acta Chim. (Budapest), 33, 121 (1962).

84. G.J. Patriarche and J.J. Lingane, *Anal. Chim. Acta*, 37, 455 (1967).
85. PDR 87 (Physicians Desk Reference), 41 ed., p. 884, E.R. Barn Hart publisher, N.J. (1987).
86. R.D. Duvoisin and R. Katz, *J. Am. Med. Ass. (JAMA)*, 206 (9) (1963).
87. L.T. Greene, *Anesth. Analg.*, 50, 222 (1971).
88. T.L. Slovis, J.E. Ott D.T. Teitelbaum, *Clin. Toxicol.*, 4, 451 (1971).
89. J.D. Cowdy, *J. Am. Med. Ass. (JAMA)*, 221, 585 (1972).
90. S. Bernstein and R. Leff, *N. Engl. J. Med.*, 227, 638 (1967).
91. W. Bernards, *Anesth. Analg.*, 52, 938 (1973).
92. G.B. Koelle and A. Gilman, "Anticholinesterase Drugs", *Pharmacol. Rev.*, 1 166 (1949).
93. Goodman and Gilman's "The Pharmacological Basis of Therapeutics", 7th ed., A.G. Gilman, L.S. Goodman, T.W. Rall and F. Murad Edits., p. 123, Macmillan Publishing Co., New York (1985).
94. J.D. Wirtschafer and W.K. Herman, *Ophthalmology (Rochester)*, 87, 1037 (1980).
95. W.H. Havener, "Ocular Pharmacology", 5th ed., C.V. Mosby Co., St. Louis (1983).
96. P.L. Kaufman, T. Weidman and J.R. Robinson, "J.R. Cholinergics, in *Pharmacology of the Eye*", M.L. Sears Edit., *Hand Book of Experimental Pharmacology*, p. 149, vol. 69, Springer-Verlag, Berlin (1984).
97. L.J. Thai, P.A. Fuld, D.M. Masur and N.S. Sharpless, "Oral Physostigmine and lucithin improve memory in Alzheimer's disease", *Ann. Neurol.*, 13, 491 (1983).
98. M.B. Walker, *Lancet*, 1, 1200 (1934).
99. D.A. Robertson, "The Calabar bean as a new agent in ophthalmic practice", *Edin. Med. J.*, 8, 815-820 (1863).

100. E. Nelson, "Physostigmine treatment in various drug-induced intoxications", *Ann. Clin. Res.*, 14, 165-172 (1982).
101. E. Stedman, "Chemical Constitution and Miotic Action", *Am. J. Physiol.*, 90, 528 (1929).
102. S.M. Aquilonius, "Physostigmine in the treatment of drug overdose, In cholinergic Mechanisms and Psychopharmacology", vol. 24, D.J. Jenden Edit., p. 817-825, Plenum Press, New York (1977).
103. B. Schwartz, "The Glaucomas", *N. Engl. J. Med.*, 290, 182-186 (1978).

## Prazosin Hydrochloride

Leonard J. Kostek  
Pfizer Inc. Central Research  
Groton, Connecticut 06340

1. Introduction
2. Description
3. Synthesis
4. Physical properties
  - 4.1 Infrared Spectrum
  - 4.2 Ultraviolet Spectrum
  - 4.3 Proton Nuclear Magnetic Resonance Spectrum
  - 4.4 Carbon-13 Nuclear Magnetic Resonance Spectrum
  - 4.5 Mass Spectrum
5. Elemental Analysis
6. Solubility
7. Polymorphic and Solvated Forms
8. Identification
  - 8.1 Thin-Layer Chromatography
  - 8.2 Infrared Absorption Spectroscopy
  - 8.3 Ultraviolet Absorption Spectroscopy
9. Methods of Analysis
  - 9.1 HPLC
  - 9.2 Potentiometric Titration
  - 9.3 Thin-Layer Chromatography
10. Pharmacokinetics and Metabolism
11. Determination in Biological Fluids
12. Bibliography

## 1. Introduction

Prazosin hydrochloride was synthesized and developed by the Medicinal Chemistry Laboratories of Pfizer Inc.<sup>1</sup> Prazosin hydrochloride was introduced as a novel antihypertensive agent with pharmacological activity attributed to arteriolar vasodilation. The antihypertensive activity was initially reported by Scriabine *et. al.*<sup>2</sup> A description of the hypotensive action of prazosin and the pharmacology of the drug was first explained by Constantine *et. al.*<sup>3</sup> A further review of the pharmacology and therapeutic efficacy of prazosin hydrochloride is given by Brogden *et. al.*<sup>4</sup> This review was updated in 1983 by Stanaszek *et. al.*<sup>5</sup> The anhydrous alpha form is the preferred polymorphic form of prazosin hydrochloride due to its non-hygroscopic characteristic leading to ease of handling and formulation. The prazosin hydrochloride alpha polymorphic form was used throughout the clinical efficacy studies. Clinical observations of prazosin hydrochloride in ambulatory patients was reported by Cohen.<sup>6</sup> This preliminary study was conducted using a crossover protocol with prazosin hydrochloride, polythiazide, prazosin hydrochloride/polythiazide combination and a placebo.

## 2. Description

### 2.1 Nomenclature

#### Chemical Name

1-(4-Amino-6,7-dimethoxy-2-quinazolinyl)-4-(2-furoyl)piperazine monohydrochloride

#### Generic Name

Prazosin Hydrochloride

#### Laboratory Code

CP-12,299-1

#### Trade Names

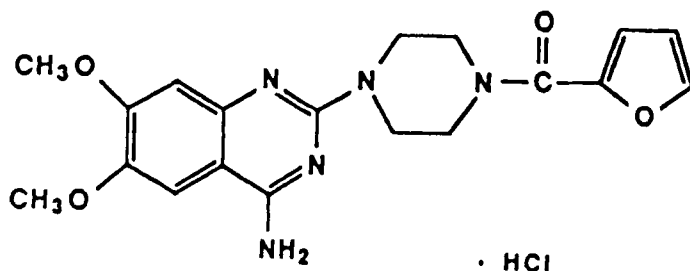
Minipress, Hypovase, Sinetens

#### CAS Registry Number

19237-84-4

## 2.2 Formula and Molecular Weight

$C_{19}H_{22}N_5O_4Cl$  Molecular Weight = 419.87



## 2.3 Appearance, Color, Odor

Prazosin hydrochloride is a white to off-white, crystalline, odorless powder

## 3 Synthesis

The synthetic process for prazosin hydrochloride has been documented in U.S. Patent #3,511,836.<sup>1</sup> The schematic representation of the synthetic process for prazosin hydrochloride is shown in Figure (1). 2-Amino-4,5-dimethoxybenzoic acid is reacted with potassium cyanate while in acetic acid forming an intermediate which is cyclized with the addition of sodium hydroxide. The reaction mixture is acidified with hydrochloric acid forming 6,7-dimethoxy-2,4-(1H, 3H)-quinazolin-2-one crystals. The dione product is refluxed with phosphorous oxychloride in N,N-dimethylaniline to produce 2,4-dichloro-6,7-dimethoxyquinazoline. Amination of the dichloro compound in tetrahydrofuran with anhydrous ammonia produces one of the intermediates 2-chloro-4-amino-6,7-dimethoxyquinazoline needed in the final coupling step to synthesize prazosin hydrochloride. The second coupling intermediate, 1-(2-furoyl)piperazine, is produced by a reaction of piperazine with hydrobromic acid and 2-furoyl chloride in an aqueous-ethanol solvent. The two immediate precursors 2-chloro-4-amino-6,7-dimethoxyquinazoline and 1-(2-furoyl) piperazine are coupled by refluxing in isoamyl alcohol to produce the desired product, prazosin hydrochloride.



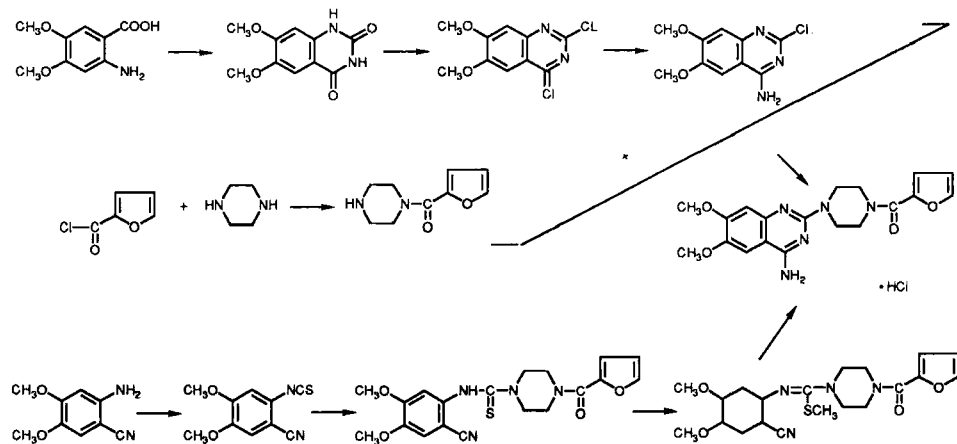


Figure (1) Synthetic Pathways for Prazosin Hydrochloride

Alternatively, Honkanen *et. al.*<sup>7</sup> describe a four step synthesis producing prazosin hydrochloride at high purity with a yield of 85-86%. The synthesis involves reaction of 3,4-dimethoxy-6-amino-benzonitrile with thiophosgene in a two-phase system to give 3,4-dimethoxy-6-isothiocyanatobenzonitrile. This benzonitrile derivative is reacted with 1-(2-furoyl)piperazine to produce 3,4-dimethoxy-6-[4-(2-furoyl)piperazine-1-yl-thiocarbamido]benzonitrile. The thiourea derivative is methylated to give methyl N-(3,4-dimethoxy-6-cyano-phenyl)-[4-(2-furoyl)piperazine-1-yl]thioformamide. This methylated thiourea derivative is reacted with ammonium chloride or urea hydrochloride causing intramolecular quinazoline ring formation and the production of prazosin hydrochloride.

#### 4. Physical Properties

##### 4.1 Infrared Spectrum

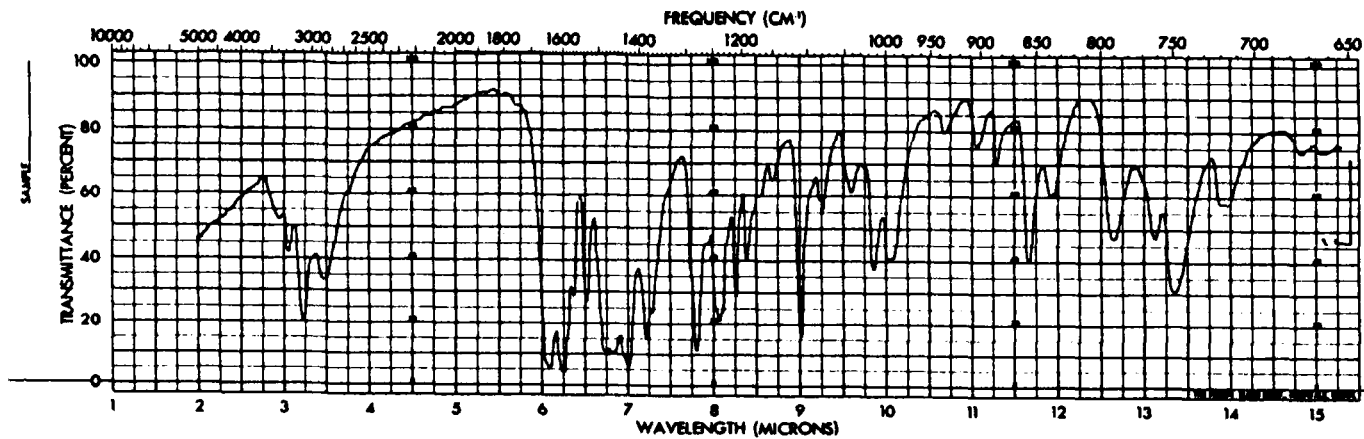
The infrared absorption spectrum of prazosin hydrochloride was obtained as a KBr pellet using a Perkin-Elmer model 21 infrared spectrophotometer. The infrared spectrum of prazosin hydrochloride is shown in Figure (2). Assignments for characteristic infrared absorption bands for prazosin hydrochloride in potassium bromide<sup>8</sup> have been listed in Table (1).

Table (1). Infrared Band Assignments for Prazosin Hydrochloride

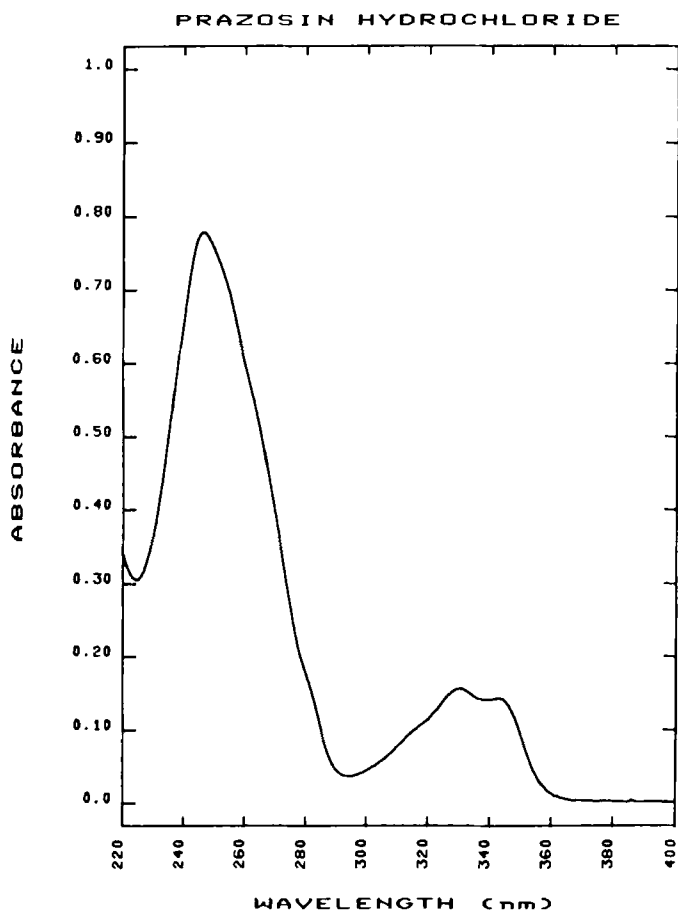
Wavelengths Microns	Assignment
6.26	Amide stretching
6.12	C = N quinazoline stretching
6.75	C = C aromatic ring stretching
7.02	hydrogen bending in piperazine ring
8.08	methoxy group vibrations
9.02	C-O-C single bond stretching in the furan ring
10.02	methoxy group vibrations

##### 4.2 Ultraviolet Spectrum

The ultraviolet absorption spectrum of prazosin hydrochloride is shown in Figure (3) at a concentration of 0.0056 mg/ml. The spectrum was obtained on a Hewett-Packard model 8450. This prazosin hydrochloride spectrum in methanolic 0.01N hydrochloric acid is characterized by two well defined maxima at 246 nm and 329 nm. The molar absorptivity values for a bulk sample of prazosin hydrochloride when determined in methanolic 0.01N hydrochloric acid were 137 +/- 3 at 246 nm and 27.6 +/- 0.3 at 329 nm. The ultraviolet absorption spectrum of



Figure(2) Infrared Absorption Spectrum of Prazosin Hydrochloride in a KBr Disc

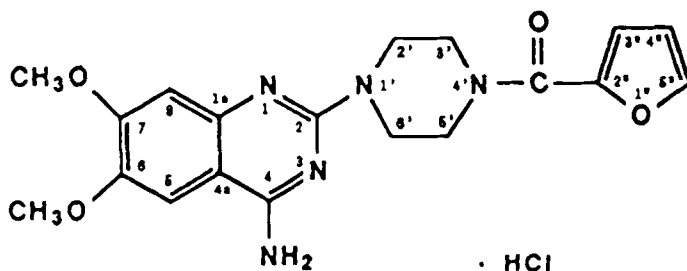


Figure(3) Ultraviolet Absorption Spectrum of Prazosin Hydrochloride at a Concentration of Approximately 0.0056 mg/ml in Methanolic 0.01 N Hydrochloric Acid

prazosin hydrochloride is representative of the additive spectra of the quinazoline and furoyl piperazine portions of the molecule.<sup>8</sup>

#### 4.3 Proton Nuclear Magnetic Resonance

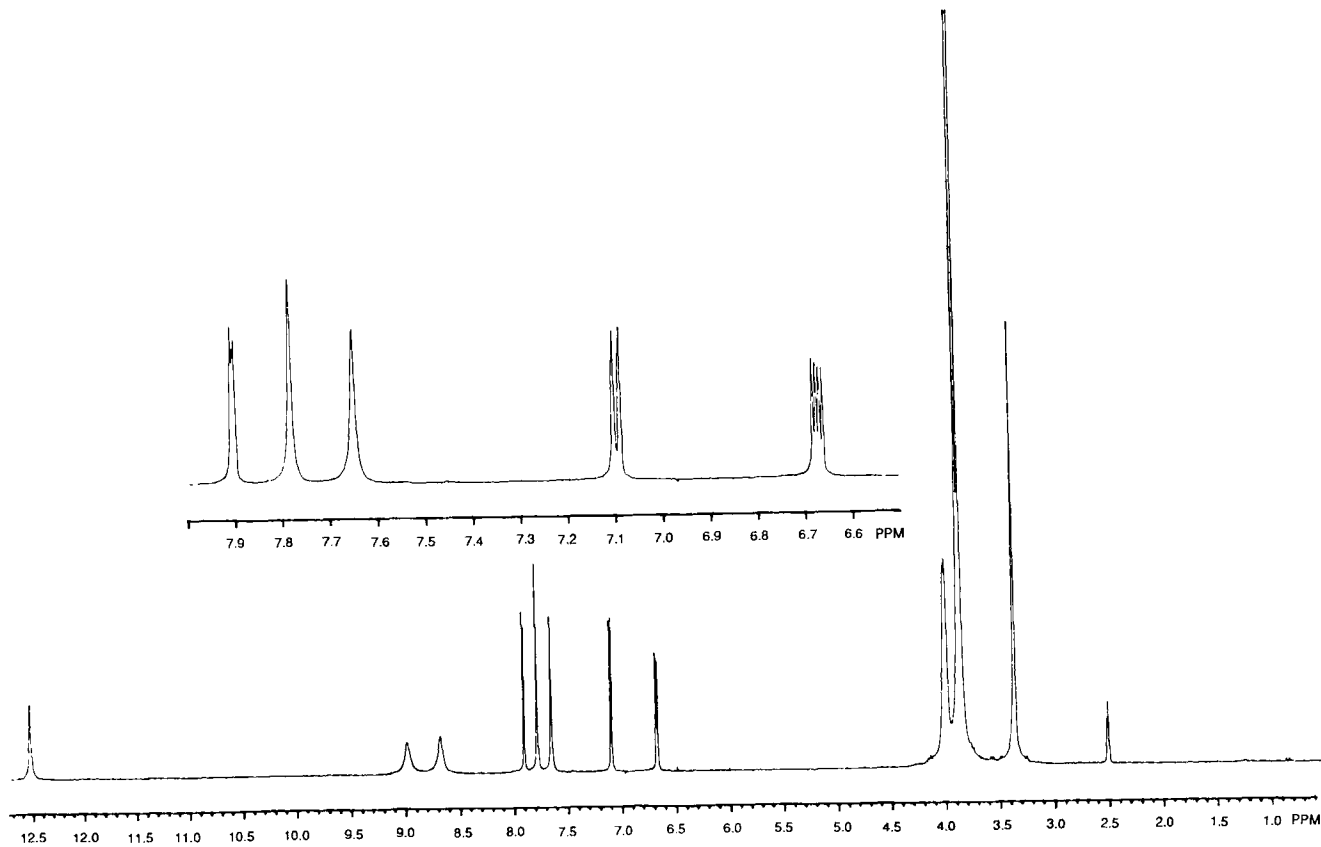
The proton magnetic resonance spectrum of prazosin hydrochloride is shown in Figure (4). The sample was run as a saturated solution in deuterated dimethylsulfoxide. The spectrum was obtained on a Bruker model 250 MHz instrument. The spectral assignments are presented below.



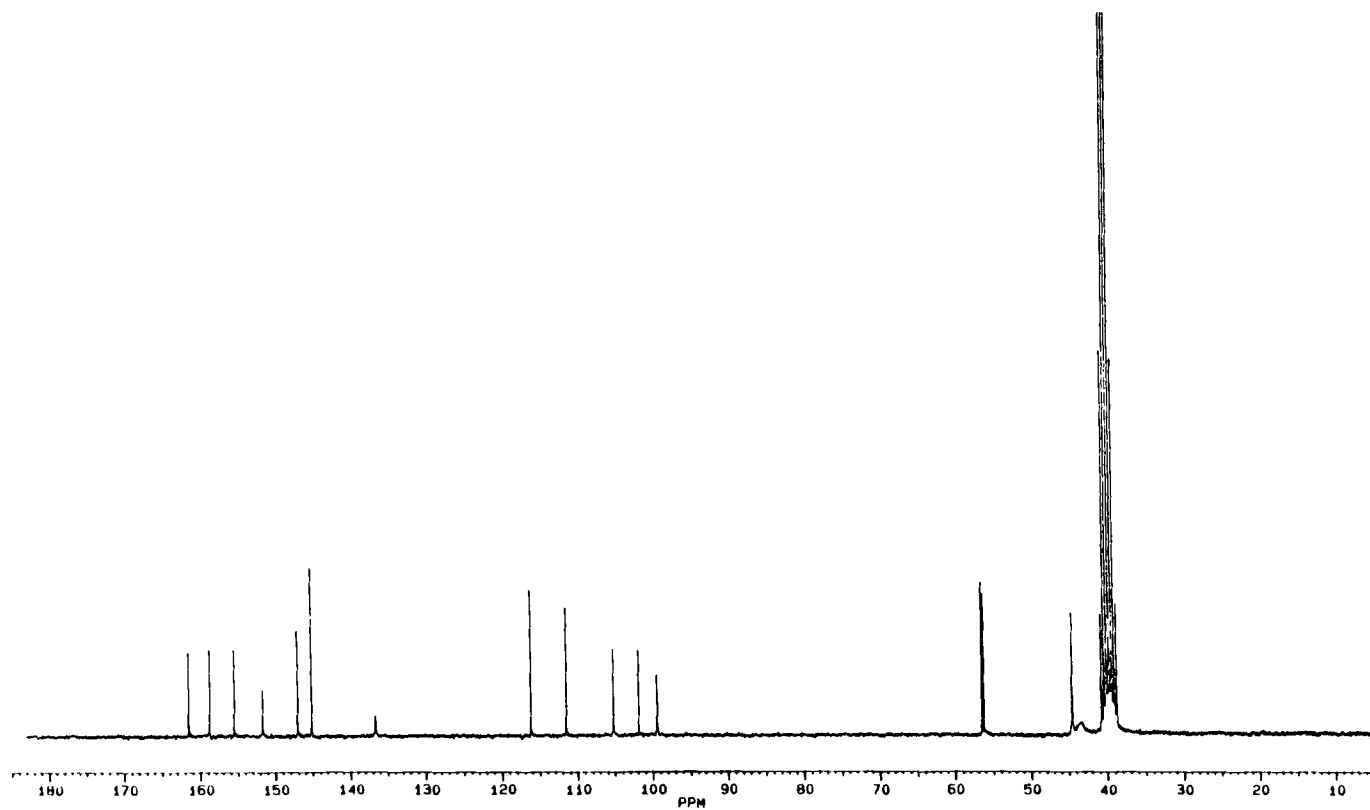
<u>Proton Assignment</u>		<u>Chemical Shift (ppm)</u>
proton in furan ring at C-5"	multiplet	7.905
aromatic proton at C-5	singlet	7.779
amide protons	singlets	8.685, 8.990
proton in furan ring at C-3"	doublet	7.096
aromatic proton at C-8	singlet	7.648
proton in furan ring at C-4"	quartet	6.664
piperazine protons	multiplet	4.002
methoxyl protons	doublet	3.876

#### 4.4 Carbon-13 Nuclear Magnetic Resonance

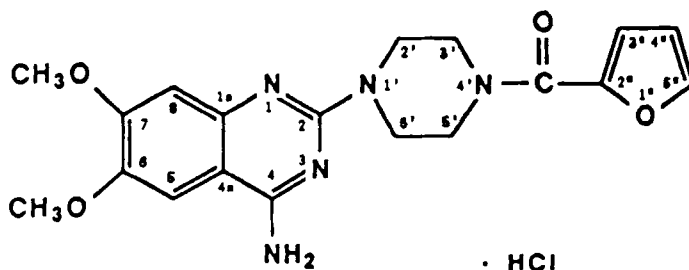
The Carbon-13 magnetic resonance spectrum of prazosin hydrochloride is shown in Figure (5). The sample was run as a saturated solution in deuterated dimethylsulfoxide. The spectrum was obtained on a Bruker model 250 MHz instrument. The spectral assignments are presented below.



Figure(4) Proton NMR Spectrum of Prazosin Hydrochloride



Figure(5) Carbon-13 Spectrum of Prazosin Hydrochloride



Chemical Shift  
(ppm)

Assignment

161.23	nonprotonated quinazoline carbon 1a
158.48	nonprotonated carbon C-2 or carbonyl
155.21	phenyl C-7
151.39	nonprotonated carbon C-2 or carbonyl
146.78	phenyl C-6
144.92	furan carbon C-5"
136.35	quinazoline carbon at amide linkage C-4
116.01	furan carbon C-3"
111.33	furan carbon C-4"
105.07	protonated phenyl carbon C-5
101.72	nonprotonated quinazoline carbon C-4a
99.28	protonated phenyl carbon C-8
56.00	methoxyl carbon at C-6 linkage
56.21	methoxyl carbon at C-7 linkage
44.39	piperazine carbons at C-2', 3', 5', 6' with some unexplained
43.30	line broadening at the lower chemical shift

The carbons at 158.48 ppm and 151.39 ppm are unassigned. These are attributed to two of the three nonprotonated carbons at C-2, C-2" and the carbonyl carbon. One carbon band is missing from the Carbon-13 spectrum. This missing band is postulated as being hidden under one of the identified bands. Further experimentation would have to be performed to find and identify the three remaining carbons.



#### 4.5 Mass Spectrum

The direct probe electron impact (EI) mass spectrum of prazosin hydrochloride as shown in Figure (6) was obtained on a VG 70/250S mass spectrometer using 70 ev electron energy and a probe temperature of 250 degrees C. The mass spectrum of prazosin hydrochloride Figure (6) shows a base molecular peak at 383 m/z. Other prominent peaks are observed at  $m/z = 288, 259, 245, 233, 205$  and 95. These fragmentation species lead to a break down pattern as suggested in Figure (7). These data are consistent with the fragmentation pattern suggested by Honkanen *et. al.*<sup>9</sup> These analyses indicate either a direct formation of the fragments from the molecular ion or additional fragmentation from the  $m/z = 288$  species.

#### 5. Elemental Analysis

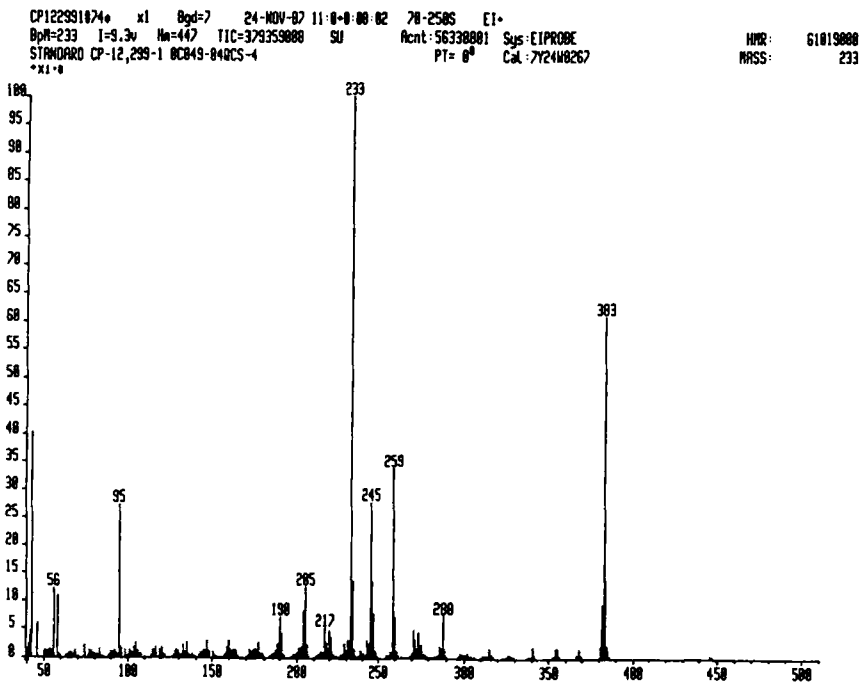
A reference sample of prazosin hydrochloride was analyzed for carbon, hydrogen, nitrogen, chloride and methoxyl content. The analysis for C, H, N was conducted using a Perkin-Elmer model 2400 elemental analyzer. The chloride content was determined using a Schrönigher combustion technique followed by titration with a standardized silver nitrate solution. The methoxyl content was determined by refluxing prazosin hydrochloride in the presence of hydroiodic acid, treating the refluxed solution with bromine and titrating with standardized sodium thiosulfate solution. The results from these determinations are given in Table (2). These data show excellent correlation with the theoretical calculated values.

Table (2). Elemental Analysis Data for Prazosin Hydrochloride

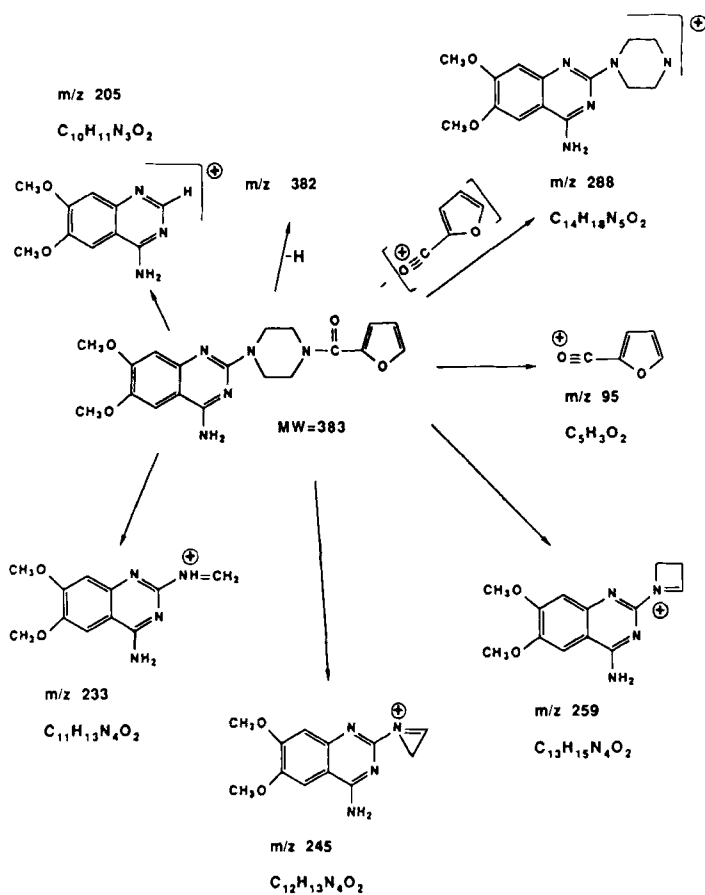
<u>Element</u>	<u>% Calculated</u>	<u>% Found</u>
C	54.35	54.34
H	5.28	5.26
N	16.68	16.49
Cl	8.44	8.42
Methoxyl	14.78	14.70

#### 6. Solubility

The following solubility data for prazosin hydrochloride (Table 3) have been obtained at ambient temperature.<sup>9</sup>



Figure(6) Electron Impact(EI) Mass Spectrum of Prazosin Hydrochloride



Figure(7) Mass Spectral Fragmentation Pattern for Prazosin Hydrochloride

**Table (3). Solubility of Prazosin Hydrochloride in Various Solvents**

<u>Solvent</u>	<u>Approximate Solubility (milligram/milliliter)</u>
acetone	0.0072
methanol	6.4
ethanol	0.84
dimethylformamide	1.3
dimethylacetamide	1.2
water (pH $\approx$ 3.5)	1.4
chloroform	0.041

These data were determined by a sample workup of saturated solutions of prazosin hydrochloride and assayed by ultraviolet absorption spectrophotometry.

## 7. Polymorphic and Solvated Forms

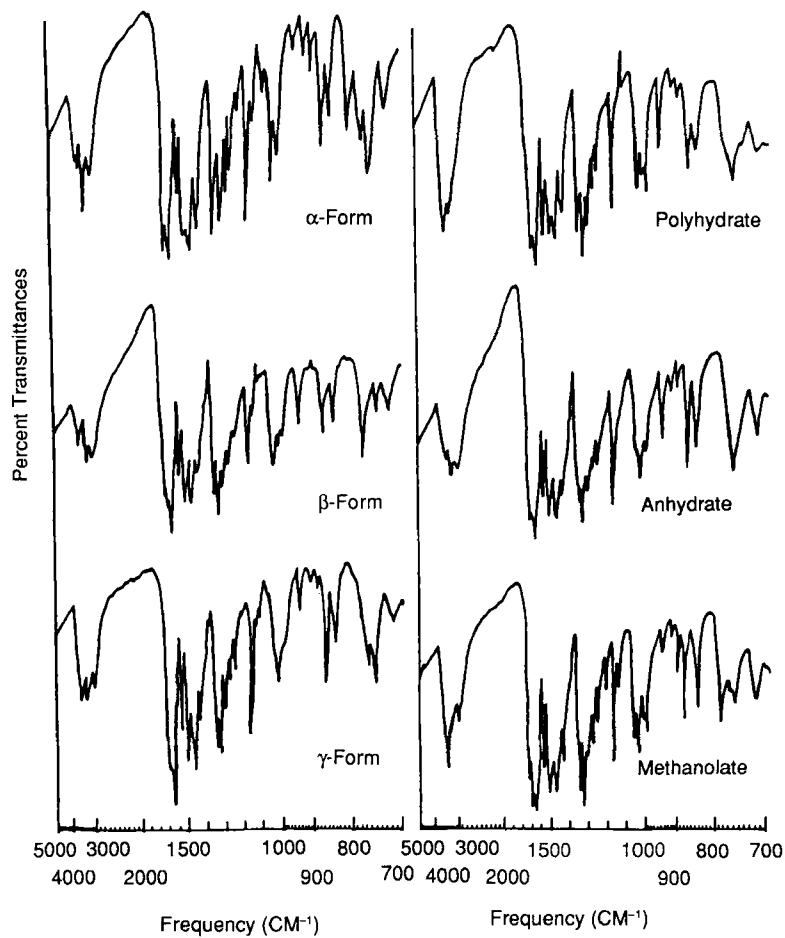
### Polymorphs

Prazosin hydrochloride is known to exist in various polymorphic forms. The alpha form is reproducibly manufactured and has valuable advantages over the other polymorphic forms due to ease of handling, storage, stability and formulating. This alpha form was used in clinical trials to show efficacy of the drug.

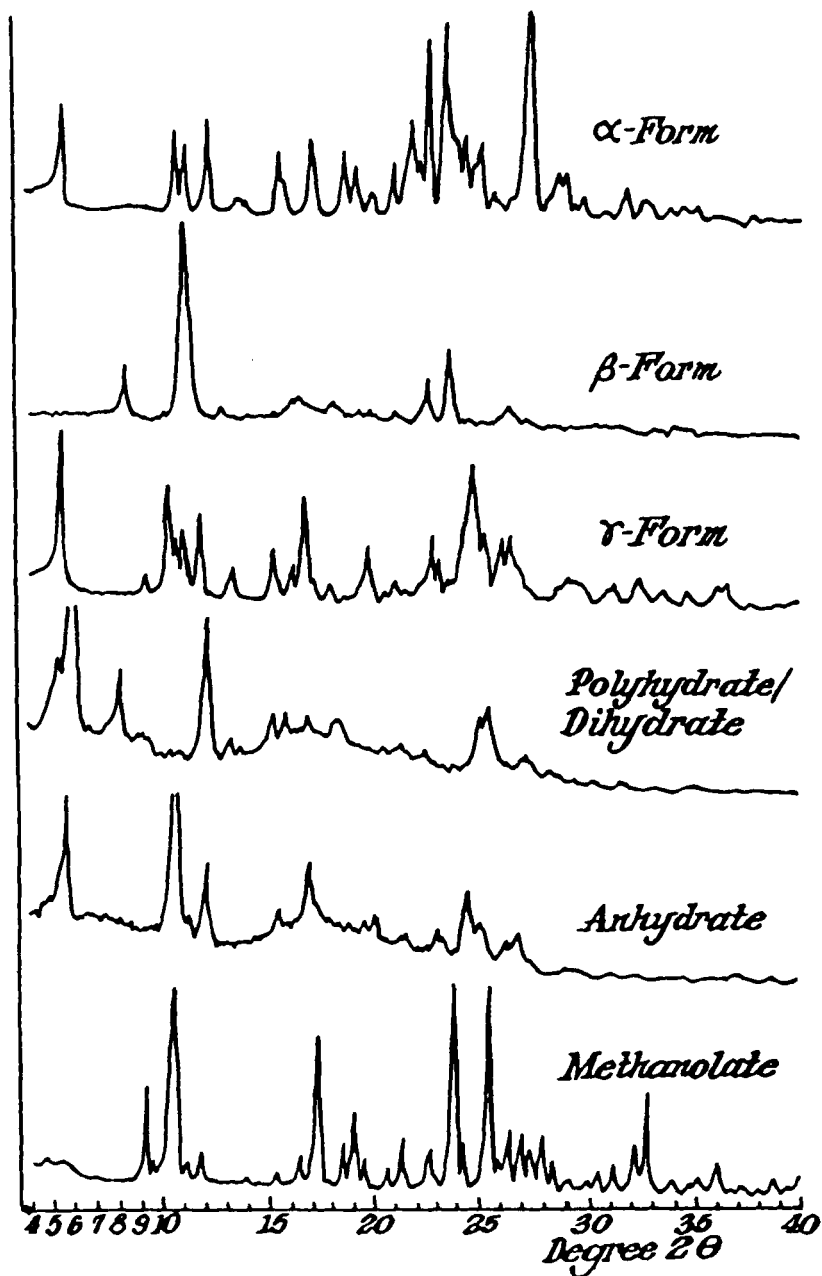
Other polymorphic forms of prazosin hydrochloride have been identified as the beta form and the gamma form. The various polymorphic forms of prazosin hydrochloride may be differentiated by using a combination of infrared (KBr pellet) spectrophotometry and powder x-ray diffraction patterns. Examples of the infrared spectra and powder x-ray diffraction patterns for these prazosin hydrochloride polymorphs are shown in Figure (8) and Figure (9) respectively. Infrared absorption bands which characterize each of the polymorphs are listed in Table (4). The characteristic powder x-ray diffraction peaks for each prazosin hydrochloride polymorph are listed in Table (5).

### Solvates

Prazosin hydrochloride is known to exist in various solvated forms. The methanolate, the anhydrate and various hydrated forms have been identified. The anhydrate is formed by drying one of the various hydrated forms to complete dryness. The various hydrated forms are prepared from aqueous slurries of prazosin hydrochloride followed by filtering and drying. Infrared absorption bands which



Figure(8) Infrared Absorption Spectra of Prazosin Hydrochloride Polymorphs and Solvates



Figure(9) Powder X-ray Diffraction Patterns for Prazosin Hydrochloride Polymorphs and Solvates

characterize each of the solvated forms are listed in Table (4). The characteristic powder x-ray diffraction peaks for each prazosin hydrochloride solvated form are listed in Table (5).

## 8. Identification

### 8.1 Thin-Layer Chromatography

The thin-layer chromatographic system used for identification of prazosin hydrochloride separates prazosin from potential contaminants. The system as described in the USP XXI<sup>10</sup> consists of silica gel GF plates, ethyl acetate:diethylamine (19.1, v/v) as developing solvent and 254 nm ultraviolet light as the detection method. The identity test is conducted with 5.0 mg/ml solutions of prazosin hydrochloride reference standard and sample. An aliquot, 0.100 ml of reference solution, approximately 2.5 inches long is applied to one half of the origin line. Similarly, 0.100 ml of the sample solution is applied to the other half of the origin line. The thin-layer plate is developed using an ascending technique until the solvent front reaches one inch of the top of the plate. The thin-layer plate is air dried, then examined under 254 nm ultraviolet light. Prazosin appears as a blue band on a yellow-green fluorescent background.

### 8.2 Infrared Absorption Spectroscopy

An infrared spectrum of prazosin hydrochloride is shown in Figure (2). This sample was obtained as a KBr pellet as described in USP XXI<sup>11</sup> for prazosin hydrochloride using a Perkin-Elmer model 21 spectrophotometer. When a sample and a reference sample are run concurrently in an identical fashion, the infrared method is a good means of identification for prazosin hydrochloride bulk. The infrared absorption technique is a good method for identification of the polymorphic form of prazosin hydrochloride. More information about the infrared identification of polymorphic forms of prazosin hydrochloride is given in the Polymorph section of this paper.

### 8.3 Ultraviolet Absorption Spectroscopy

The ultraviolet absorption spectrum of prazosin hydrochloride obtained in methanolic 0.01N hydrochloric acid is shown in Figure (3). This spectrum exhibits characteristic absorption maxima at 329 nm, and 246 nm and absorption minima at 295 nm and 224 nm. The spectrum shown in Figure (3) was obtained with a Hewlett-Packard model 8450 dual beam ultraviolet/visible spectrophotometer using one centimeter cells at a concentration of approximately 0.0056 mg/ml. The characteristic ultraviolet absorption spectrum is an excellent test for identi-

**Table 4.      Characteristics Infrared Absorption Bands for Prazosin Hydrochloride Polymorphic and Solvated Forms**

<u>Polymorphic Form</u>	<u>Characteristic Bands</u>		
	<u>cm<sup>-1</sup></u>	<u>microns</u>	<u>band shape</u>
alpha	795	12.6	sharp
beta	770	13	sharp
gamma	770	13	doublet
	743	13.4	doublet
<u>Solvated Form</u>			
polyhydrate*	1260	7.95	sharp
	755	13.3	broad
	1000	10	doublet
anhydrate	1260	7.95	sharp
	755	13.3	broad
	1005	9.95	triplet
methanolate	870	11.5	sharp

\* Infrared spectra of all hydrated forms are identical.



**Table 5. Characteristics Powder X-ray diffraction peaks for Prazosin Hydrochloride Polymorphic and Solvated Forms**

<u>Polymorphic Form</u>	<u>Characteristic Peak</u>
alpha	1:3:3:1 quartet centered at 23 degrees sharp bands at 93 degrees and 23.6 degrees
beta	sharp bands at 11.3 degrees, 22.5 degrees and 23.6 degrees
gamma	band groupings at 10.5 degrees, 16.7 degrees and 24.8 degrees
<u>Solvated Form</u>	
polyhydrate	sharp bands at 8.05 degrees, 12.2 degrees doublet at 25.2 degrees
anhydrate	same as monohydrate form sharp bands at 10.5 degrees, 12.0 degrees and 16.9 degrees doublets at 24.5 degrees, 26.5 degrees
methanolate	sharp bonds at 10.5 degrees, 17.3 degrees, 23.9 degrees, and 25.5 degrees

fication of a prazosin hydrochloride sample to that of a reference standard when the two spectra are determined concurrently in an identical fashion as described in the USP XXI<sup>11</sup>.

## 9. Methods of Analysis

### 9.1 High Performance Liquid Chromatography (HPLC)

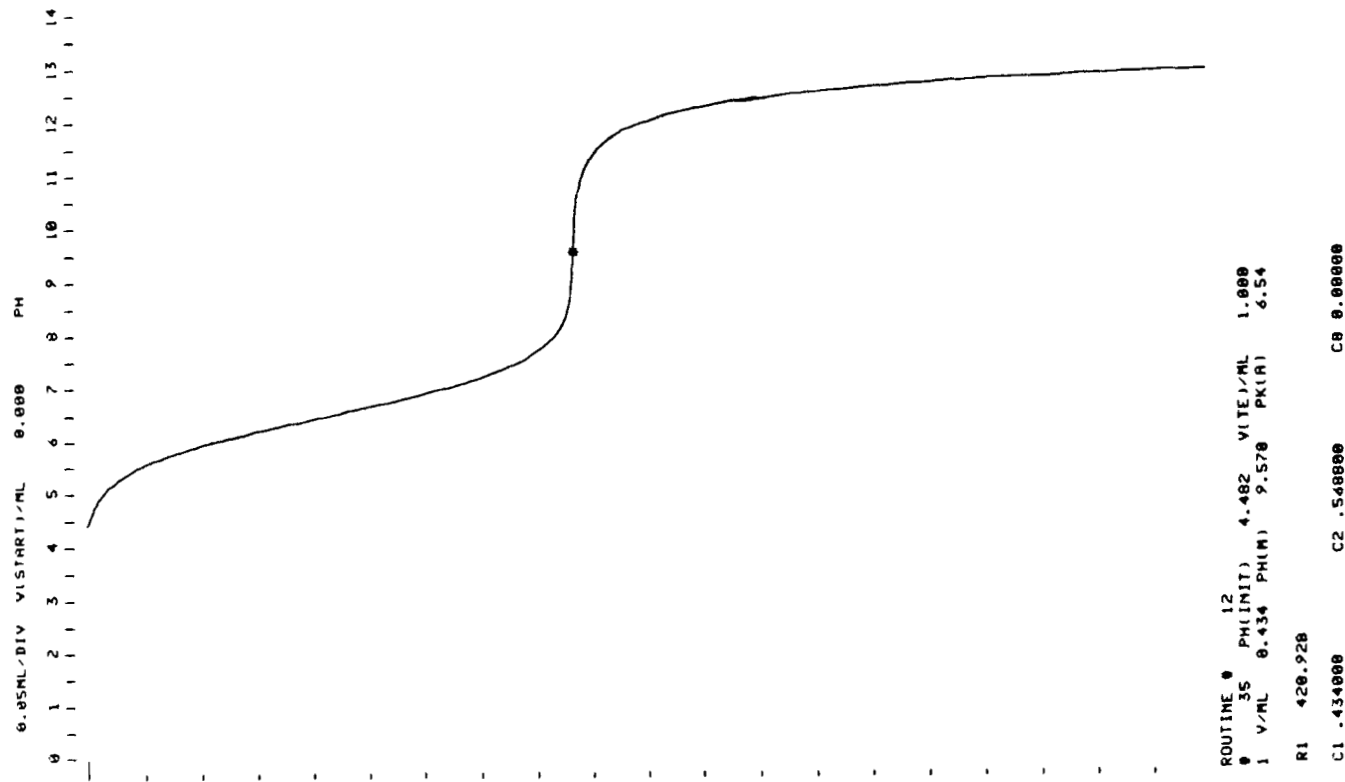
Essentially one HPLC method has been developed for the determination of prazosin in prazosin hydrochloride bulks. The assay method for prazosin in bulk samples of prazosin hydrochloride as found in USP XXI<sup>12</sup> uses a Zorbax-SIL column from DuPont, 254 nm UV detection, 0.2 ml flow rate and a mobile phase consisting of Methanol:Water:Acetic Acid (7:2:1, v/v) with diethylamine added. Enough diethylamine per liter (2 to 25 drops) is added to give a retention time of 6 to 10 minutes for prazosin. This system separates prazosin from potential impurities, and possible degradation products.

### 9.2 Potentiometric Titration

Prazosin hydrochloride is assayed potentiometrically by dissolving the sample in ethanol:water (1:1, v/v) and titrating with standard aqueous sodium hydroxide and a combination pH electrode is used for the potentiometric system. The theoretical neutral equivalent for prazosin hydrochloride is 419.87. When a reference standard of prazosin hydrochloride was assayed by this potentiometric procedure, a value of 420.9 was obtained for the neutral equivalent. The pKa value for this reference standard determined in ethanol:water (1:1, v/v) was determined to be 6.54. A typical potentiometric curve for prazosin hydrochloride obtained on a Metrohm model E636 autotitrator is presented in Figure (10).

### 9.3 Thin-Layer Chromatography

Prazosin hydrochloride is analyzed for potential impurities by a thin-layer chromatographic procedure as described in the USP XXI<sup>12</sup> and USP XXI Supplement 7<sup>13</sup>. The analysis involves three thin-layer chromatographic systems and two detection methods. The method is capable of separating prazosin hydrochloride from small amounts of five potential impurities. Also, the procedure can establish the level of these potential impurities, if present, in the prazosin hydrochloride sample. The chromatographic procedure uses silica gel GF (Merck-Darmstadt) plates as the solid phase adsorbent. The three developing solvents and the two detection methods are listed in Table (6). The prazosin hydrochloride sample is prepared at a concentration of approximately



Figure(10) Potentiometric Titration of Prazoin Hydrochloride

12 mg/ml in chloroform:methanol:diethylamine (10:10:1, v/v), the dissolving solvent. The prazosin solution and potential impurity solution are applied to the thin-layer plate in 0.100 ml aliquots. The prazosin hydrochloride sample is applied to the origin line as a three inch streak to one side of the thin-layer plate. The potential impurity is applied as a three inch streak to the other side of the thin-layer origin line so that it overlaps the prazosin hydrochloride streak by one inch. The TLC plates are developed using an ascending technique until the solvent front reaches within one inch of the top edge of the plate. The potential impurities, developing solvent systems and relative mobilities of prazosin hydrochloride and the potential impurities are presented in Table (6). Using a combination of solvent systems as described in Table (6), a potential impurity may be separated from prazosin hydrochloride and the other potential impurities.

**Table 6. Potential Impurities, Relative Mobilities, Detection Methods and Solvent Systems**

Compound	Relative Mobilities in Solvent Systems*			Detection Method
	(a)	(b)	(c)	
(1) 2-Chloro-4-amino-6,7-dimethoxyquinazoline	0.27	0.65	0.40	1
(2) 1-(2-Furoyl)piperazine	0.00	0.33	0.15	2
(3) 1,4-Bis-(4-amino-6,7-dimethoxy-2-quinazolinyl)-piperazine	0.00	0.70	0.25	1
(4) 4-Amino-6,7-dimethoxy-2-(1-piperazinyl)-quinazoline dihydrochloride trihydrate	0.00	0.25	0.05	1
(5) 1,4-Bis-(2-furoyl)piperazine	0.18	0.73	0.80	1
(6) Prazosin Hydrochloride	0.00	0.75	0.48	1

\*Solvent system (a) = toluene-ethyl acetate-acetic acid (10:10:1, v/v).

\*Solvent system (b) = ethyl acetate-methanol-diethylamine (40:20:3, v/v).

\*Solvent system (c) = ethyl acetate-diethylamine (19:1, v/v).

Detection Method (1) = ultraviolet light – 254 nm.

Detection Method (2) = iodoplatinic acid reagent.

## 10. Pharmacokinetics and Metabolism

The pharmacokinetics and metabolism of prazosin hydrochloride have been studied by numerous investigators. Early studies by Wood<sup>14</sup> using normotensive volunteers (six male and four female) given a single (5 mg) dose after overnight fasting found peak plasma levels at 2-3 hours after oral administration. Plasma concentrations followed first order kinetics with a half-life of 3.9 hours. Likewise, Collins<sup>15</sup> found significant first pass liver metabolism of prazosin hydrochloride. Five hypertensive volunteers were given a single dose of between 2 mg and 5 mg of prazosin hydrochloride after fasting. Plasma half-life was determined to be at 3.28 hours and mean liver extraction to be approximately 29.9%.

Taylor *et. al.*<sup>16</sup> performed radiolabeled prazosin hydrochloride studies in rats and dogs. Within 30 minutes prazosin was found distributed into the lung, heart and vascular tissues of the dog. Urinary excretion of prazosin by rats and dogs was low with the major route of elimination by biliary secretion through the feces. Bioavailability of prazosin in beagle dogs was found to be dose-dependent by Baughman *et. al.*<sup>17</sup> Metabolism in the rat and dog was found to occur primarily by 6- or 7-O-dealkylation with subsequent glucuronide formation. To a lesser extent, N-dealkylation was observed with the piperazine ring opening. Studies in man with unlabeled prazosin hydrochloride produced similar metabolites to those found in the rat and dog. A further review of prazosin pharmacokinetics has been summarized by Siedman<sup>18</sup> along with the effects of an alpha-adrenoceptor blockade, with special reference to prazosin, in the treatment of hypertension.

Bateman *et. al.*<sup>19</sup> studied the pharmacokinetic effects of prazosin hydrochloride after oral and intravenous dosing using erect and supine volunteers. Intravenous and oral dosing each produced a mean terminal (beta) half-life of 2.9 hours with oral bioavailability at 56.9%. There was a greater effect in blood pressure after intravenous rather than oral dosing plus a significant correlation between blood pressure lowering and drug plasma concentration after intravenous administration. Other prazosin hydrochloride studies have been conducted to describe pharmacokinetics in man by Hobbs *et. al.*<sup>20</sup>, the effects of food on prazosin hydrochloride intake by Verbesselt *et. al.*<sup>21</sup> and plasma levels, blood pressure and heart rate by Wood *et. al.*<sup>22</sup> An additional study by Hobbs and Twomey<sup>23</sup> investigated the protein binding properties of prazosin. They found that approximately 92 percent of the prazosin was bound to serum proteins whether prazosin was administered separately or in conjunction with other frequently administered drugs.

## 11. Determination in Biological Fluids

A number of high-performance liquid chromatography (HPLC) assay methods have been developed to study prazosin in biological

fluids. Yee *et. al.*<sup>24</sup> describe a two step extraction procedure followed by an ion-pairing HPLC method with fluorescence detection. Levels of detection were at 0.2 ng/ml in whole blood and 0.5 ng/ml in plasma. Other interfering drugs were separated by slightly altering the mobile phase composition. Reece<sup>25</sup> uses a single extraction procedure to recover prazosin from biological fluids followed by reversed-phase HPLC and fluorescence detection. This system separates prazosin from a variety of drugs such as propranolol, 4-hydroxypropranolol, quinidine and various metabolites of the tested drugs. The limit of detection for prazosin was determined to be 0.015 ng/ml. An extraction method followed by dual HPLC procedures by Dokladalova *et. al.*<sup>26</sup> gives a useful method for determining prazosin blood plasma levels in the presence of polythiazide, a diuretic frequently administered in combination with prazosin hydrochloride. The prazosin assay requires reversed-phase chromatography with fluorescence detection. Bhamra *et. al.*<sup>27</sup> describe an HPLC method that separates and measures the levels of prazosin and terazosin in biological fluids. Terazosin is similar in structure to prazosin, having a tetrahydrofuroyl group in place of the prazosin furoyl moiety, plus both drugs are administered at low doses in treating hypertension. Piotrowskii *et. al.*<sup>28</sup> describe an ion-exchange HPLC method that separates propranolol, nadolol and prazosin in biological fluids. This method has been applied to pharmacokinetic studies to separate and assay these drugs in serum, urine and saliva.

## 12. Bibliography

- (1) US Patent #3,511,836 to Dr. Hans-Jurgen Hess, Pfizer Inc., May 1970.
- (2) Scriabine, A., Constantine, J. W., Hess, H. -J., and McShane, W. K. "Pharmacological Studies with Some New Antihypertensive Aminoquinazolines." *Experientia*, 24, 1968, p. 1150.
- (3) Constantine, J. W., McShane, W. K., Scriabine, A. and Hess, H. - J. Hypertension: Mechanisms and Management. Editors: Onesti, G., Kim, K. E. and Moyer, J. H., p. 429. New York: Grune and Stratton, Inc., 1973.
- (4) Brogden, R. N., Heel, R. C., Speight, T. M., Avery, G. S. "Prazosin: A Review of its pharmacological properties and therapeutic efficacy in hypertension." *Drugs*, 14, 1977, P. 163.
- (5) Stanaszek, W. F., Kellerman, D., Brogden, R. N. and Romankiewicz, J. A., "Prazosin Update: A review of its pharmacological properties and therapeutic use in hypertension and congestive heart failure." *Drugs*, 25, 1983, 333-384.
- (6) Cohen, B. M., "Prazosin hydrochloride (CP-12,299-1), and oral anti-hypertensive agent: preliminary clinical observations in ambulatory patients." *J. Clinical Pharmacology*, 1970, 10, p. 408.
- (7) Honkanen, E., Taskinen, J., Koivisto, M. and Nore, P., "New practical synthesis of prazosin." *J. Het. Chem.*, 17, June 1980, p. 797-798.
- (8) Finegan, R. L. and Forcier, G. A., Pfizer Inc., personnel communication, 1972.
- (9) Honkanen, E., Taskinen, J., Koivisto, M. and Nore, P., "Mass spectrometric fragmentation of prazosin and synthesis of its deuterium labelled derivation." *Finn. Chem. Lett.*, 1, 1979, p.20-22.
- (10) USP XXI, 1985, p. 868-869.
- (11) USP XXI, 1985, P. 868.
- (12) USP XXI, 1985, P. 869.
- (13) USP XXI, Supplement 7, 1988, P. 2820.
- (14) Wood, A. J., "Pharmacokinetics of prazosin in man." *Clin. and Exp. Pharmacol. and Physiol.*, 2, 1975, p. 446.
- (15) Collins, I. S., "Pharmacokinetics of prazosin, a new antihypertensive compound." *Clin. and Exp. Pharmacol. and Physiol.* 2, 1975, p. 445.
- (16) Taylor, J. A., Twomey, T. M. and Wittenau, M. S. V., "The metabolic fate of prazosin." *Xenobiotica*, 7, No. 6, 1977, p. 357-364.
- (17) Baughman, R. A. Jr., Sorgel, F., Mico, B. A. and Benet, L. Z., "Dose-dependent bioavailability of prazosin in beagle dogs." *J. of Cardiovascular Pharm.*, 5, 1983, p. 613-617.



- (18) Seideman, P., "Pharmacokinetic and dynamic aspects of alpha-adrenoceptor blockade in hypertension." Acta Med. Scand., Suppl., 1982, 665, p. 61-66.
- (19) Bateman, D. N., Hobbs, D. C., Twomey, T. M., Stevens, E. A. and Rawlins, M. D., "Prazosin, pharmacokinetics and concentration effect." Eur. J. Clin. Pharmacol. 16, 1979, p. 177-181.
- (20) Hobbs, D. C., Twomey, T. M. and Palmer, R. F., "Pharmacokinetics of prazosin in man." J. Clin. Pharmacol., 18, 1978, p. 402-406.
- (21) Verbessett, R., Mullie, A., Tjandramage, T. B., DeSchepper, P. J. and Dessain, P., "The effect of food intake on the plasma kinetics and toleration of prazosin." Acta Ther., 2, 1976, p. 27.
- (22) Wood, A. J., Bolli, B. and Simpson, F. O., "Prazosin in normal subjects: plasma levels, blood pressure and heart rate." Br. J. Clin. Pharmacol., 1, 1976, p. 199.
- (23) Hobbs, D. C. and Twomey, T. M., "Protein binding properties of prazosin." Chem. Path. and Pharmacol., 25, No. 1, 1978, p. 188-192.
- (24) Yee, Y. G., Rubin, P. C. and Meffin, P., "Prazosin determination by high-pressure liquid chromatography using fluorescence detection." J. Chrom., 172, 1979, p. 313-318.
- (25) Reece, P. A., "Quantification of prazosin in plasma by high-performance liquid chromatography." J. Chrom., 221, 1980, p. 188-192.
- (26) Dokladalova, J., Coco, S. J., Lemke, P. R., Quercia, G. T. and Korst, J. J., "Determination of polythiazide and prazosin in human plasma by high-performance liquid chromatography." J. Chrom., 224, 1981, p. 33-41.
- (27) Bhamra, R. K., Flanagan, R. J. and Holt, D. W., "High-performance liquid chromatographic measurement of prazosin and terazosin in biological fluids." J. of Chrom., 380, 1986, p. 216-221.
- (28) Piotroskii, V. K., Belolipetskaya, V. G., El'Man, A. R. and Metelitsa, V. I., "Ion-exchange high-performance liquid chromatography in drug assay in biological fluids." J. Chrom., 278, 1983, p. 469-474.

**TETRACAINE HYDROCHLORIDE**

**MOHAMMAD RIAZ\*, COLLEGE OF PHARMACY,  
UNIVERSITY OF MICHIGAN ANN ARBOR, MI 48109.**

**\* PRESENT ADDRESS: FACULTY OF PHARMACY,  
UNIVERSITY OF THE PUNJAB, LAHORE, PAKISTAN.**

## 1. Description

### 1.1 Chemical Name

2-Dimethylaminoethyl 4-n-butylaminobenzoate hydrochloride(1)  
 4-(Butylamino)benzoic acid 2-(dimethylamino)ethyl ester hydrochloride(1)  
 p-Butylaminobenzoyl-2-dimethylaminoethanol hydrochloride(1)  
 Benzoic acid, 4-(butylamino)-2-(dimethylamino)ethyl ester, monohydrochloride(2)

### 1.2 Generic Name(2)

Tetracaine hydrochloride, amethocaine hydrochloride

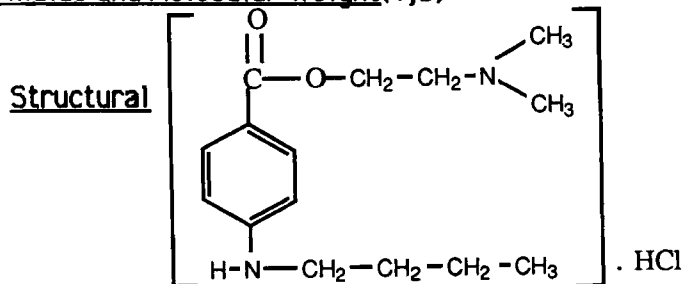
### 1.3 Trade Name

Pontocaine hydrochloride, Anestaron(2), Anethaine(3)

### 1.4 Chemical Abstract Registry Number (4) [ 136-47-0 ]

### 1.5 Wiswesser Line Notation(4) 4MR DV02NI &1 &GH

## 2. Formulae and Molecular Weight(1,5)



Empirical $C_{15}H_{24}N_2O_2 \cdot HCl$  $C_{14}H_{25}N_2O_2Cl$ Molecular Weight

300.83

3. Appearance, Color, Odor(2)

It occurs as a fine, crystalline, hygroscopic, odorless, powder which has a slightly bitter taste followed by a sense of numbness.

4. Elemental Composition(1)

C	59.89 %	Cl	11.78 %	H	8.38%
N	9.31 %	O	10.64 %		

5. Physical Properties5.1 Loss On Drying(5)

When dried to a constant weight at 100 to 105 °C, the drug loses no more than 1 % of its weight.

5.2 Solubility(6)

The drug is soluble at 20 °C in 7.5 parts of water, 40 parts of alcohol and 30 parts of chloroform; practically insoluble in ether and acetone.

5.3 Acidity

A one percent solution has a pH of 4.5 to 6.5(6). A 1 % solution of the drug was found to have a pH of 5.86.

5.4 Dissociation Constant(2)

Tetracaine hydrochloride has a pKa value of 8.39.

5.5 Clarity and Color of Solution(5)

A 10 % w/v solution is clear and colorless.

## 5.6 Surface Activity(7)

The free energy ( $\Delta G^0$ ) of adsorption of tetracaine hydrochloride at air-0.15M sodium chloride interface was found to be 7.3 kcal/mole. Further interaction energies of the drug with dipalmitoylphosphatidylethanolamine and dipalmitoyl lecithin monolayers, previously spread at the air-0.15M sodium chloride interface have been reported to be 6.5 kcal/mole for both lipids.

## 5.7 Self Association(8)

Attwood and Fletcher studied the self association of tetracaine hydrochloride in water and electrolyte solutions using total intensity light scattering, photon correlation spectroscopy, and vapour pressure osmometry techniques. They suggested that the association of the drug could be described using a cooperative stepwise association model. The critical micelle concentration of amethocaine hydrochloride in 0.4 M sodium chloride was  $6-7 \times 10^{-2}$  mol/Kg.

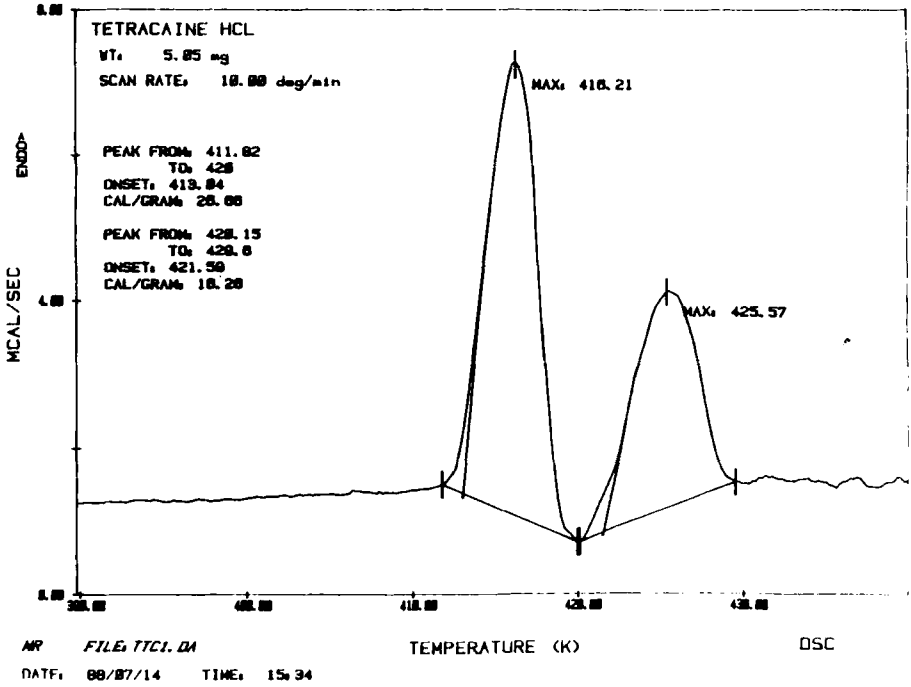
## 5.8 Melting Point(6)

It melts at about 148 °C, or it may occur in either of two polymeric forms, melting at about 134 ° and 139 °C respectively; the mixture of the polymeric forms melts within the range 134 ° to 147 °C.

## 5.9 Differential Scanning Calorimetry

Tetracaine hydrochloride was heated from 390 ° to 440 °K at a rate of 10 degree/minute under an atmosphere of nitrogen in a Perkin-Elmer Model DSC-2 Differential Scanning Calorimeter equipped with a Data Station. The presence of polymorphism can be seen by the existence of two endotherms in the thermogram (See Fig. 1). Indium was used as a reference standard. The corrected onset temperature for the two endotherms (polymeric forms) was 413.04 ° to 421.59 °K (136.5

Fig 1 DSC scan



$^{\circ}$  and 146.3  $^{\circ}$ C). Further corrected heats of fusion were 26.12 and 15.93 cal/gram for the two polymorphs.

### 5.10 Ultraviolet Absorption Spectrum

The absorption spectrum of tetracaine hydrochloride in water obtained on a Perkin Elmer Lambda 7 spectrophotometer is given in Fig. 2. The spectral data is presented in Table 1.

Table I

$\lambda_{\text{max}}$ (nm)	Solvent	E(1%, 1cm)	E
225,310	H <sub>2</sub> O	469,876	14108, 26352
229,281,312	0.1N H <sub>2</sub> SO <sub>4</sub>	509,55,76*	---
226,310	CH <sub>3</sub> OH**	---,---	7586, 29512
308	CHCl <sub>3</sub> *	----	27542

\*, \*\*, \* from Ref 3, 4, 4 respectively; --- not given.

### 5.11 Infrared Absorption Spectrum

The FT-IR spectrum of tetracaine hydrochloride was obtained with a Nicolet 5DXB/5SXB Spectrometer. A chloroform solution of the drug using a cell of path length of 0.05 mm was scanned from 4000 to 400  $\text{cm}^{-1}$ . The spectrum(after subtraction of chloroform spectrum) is presented in Fig.3. The spectral assignment of bands at 1610, 1687, 2966 and 3422  $\text{cm}^{-1}$  is given in Table 2. Peaks are also observed at 1175, 1310, 1404, 1808, 1850, 2324  $\text{cm}^{-1}$ .

Wave Number ( $\text{cm}^{-1}$ )	Assignment
1610	Benzene ring C=C stretching

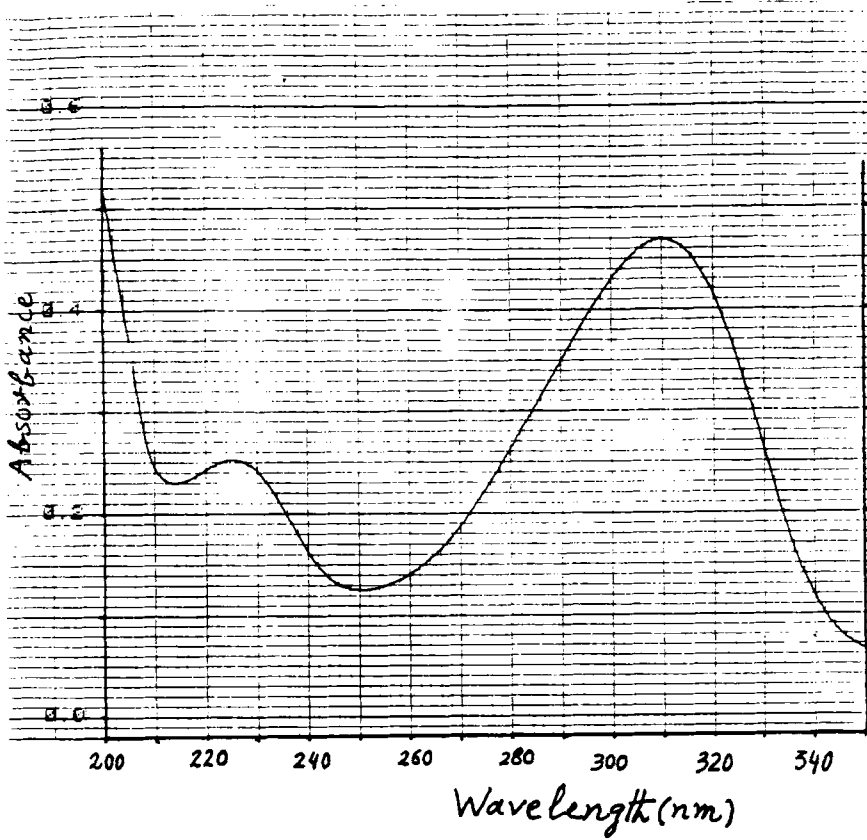


Fig 2 UV absorption spectrum



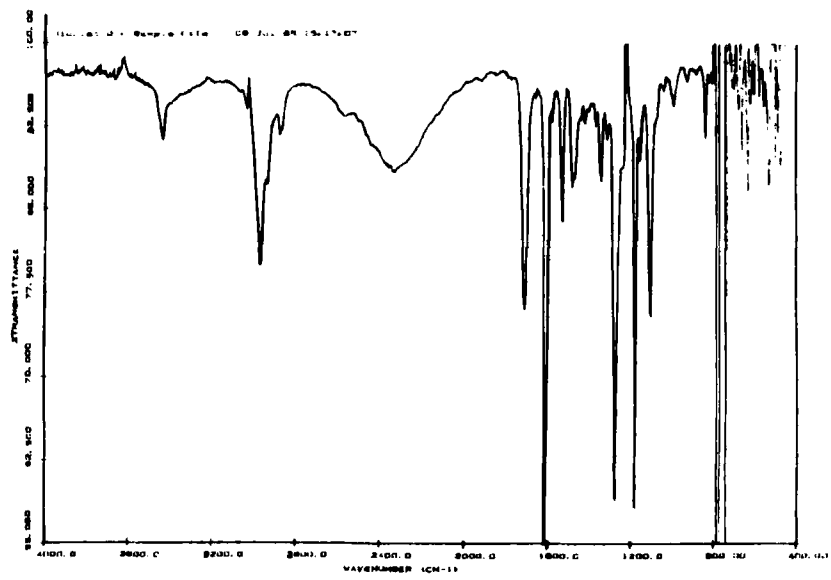


Fig 3 FT-IR absorption spectrum

1687	Stretching of C=O group in aryl ester
2966	Benzene ring C-H stretching
3422	N-H Stretching

---

The infrared spectrum of tetracaine hydrochloride(KBr disk) has been reported and six principal peaks within 2000-650  $\text{cm}^{-1}$  occur at 1600, 1286, 1174, 1688, 1126, 1532  $\text{cm}^{-1}$  (6).

### 5.12 X-ray Diffraction(9)

The x-ray powder diffraction data for tetracaine hydrochloride obtained on Norleco x-ray diffraction unit are given in Table 3. The instrument settings used during the experiment are given below.

Excitation: Emission from Cu tube(1.54  $\text{\AA}$ )  
operated at 35 kv and at 18 ma.

Slits: Divergence 1 degree, Receiving  
0.03 inch, Scatter 1 degree.

Scanning Rate: 1 degree per minute

$2\theta$	$I/I_0$	$2\theta$	$I/I_0$
1.95	4	3.78	10
2.15	4	3.97	11
2.51	5	4.22	20
2.66	5	4.43	8
2.80	7	4.83	12
2.96	6	5.05	67
3.12	10	5.42	8
3.30	10	6.28	12
3.48	17	8.38	42
3.56	8	12.55	100

---

The highest  $I/I_0$  value is taken as 100 and other values are relative to this value.

### 5.13 Proton Magnetic Resonance Spectrum

The FT-PMR spectrum of tetracaine hydrochloride in heavy water obtained on Bruker(WM360) NMR spectrometer is given in Fig. 4. The experimental conditions used during the experiment were:

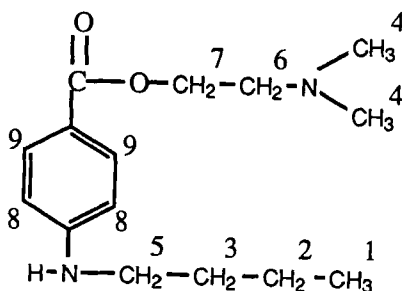
Spectrometer frequency: 360 MHz

Pulse width: 7.0  $\mu$ s

Acquisition time: 3.27 s

Number of scans: 107

The spectral assignment for PMR spectrum is given in Table 4.



Proton position	Chemical Shift (ppm)	Number of protons	Multiplicity
1	0.8443	3	triplet
2	1.3051	2	sixtet
3	1.4937	2	quintet
4	2.9829	6	singlet
5	3.0456	2	triplet
6	3.5285	2	multiplet
7	4.5188	2	multiplet
8	6.6282	2	doublet
9	7.7641	2	doublet
4.7422(residual protons present in D <sub>2</sub> O)			

### 5.14 C-13 Nuclear Magnetic Resonance Spectrum

The broad band proton decoupled and off resonance proton decoupled FT-C13 NMR spectra of tetracaine hydrochloride on Bruker(WM360) NMR spectrometer at 90 MHz in

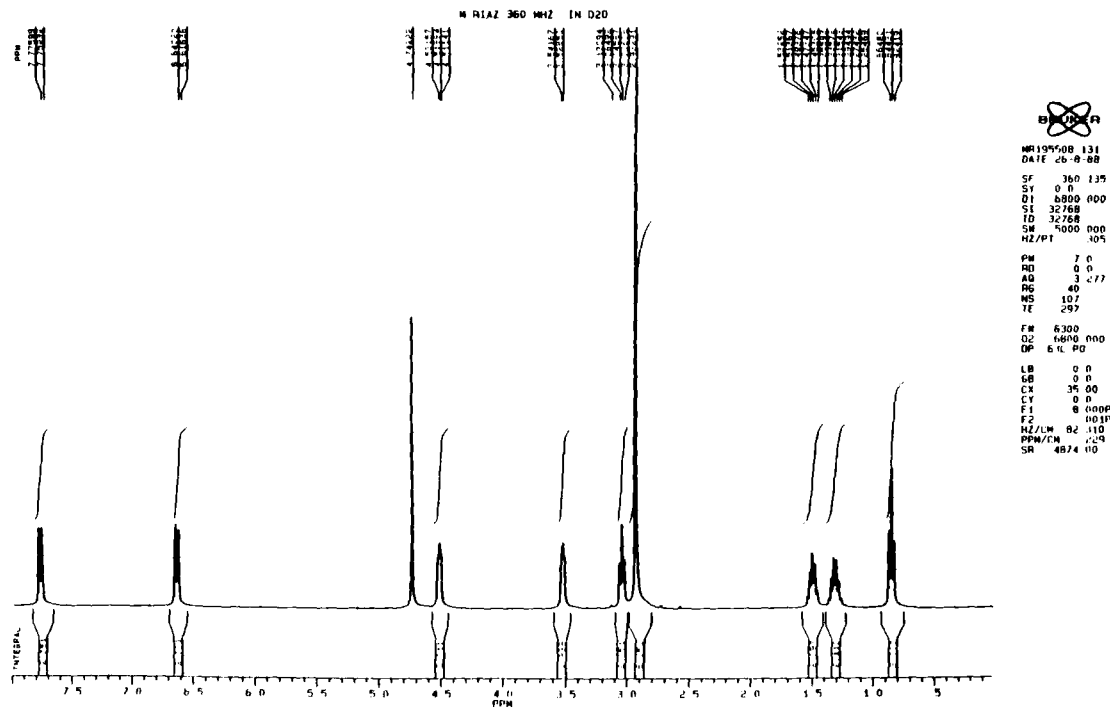
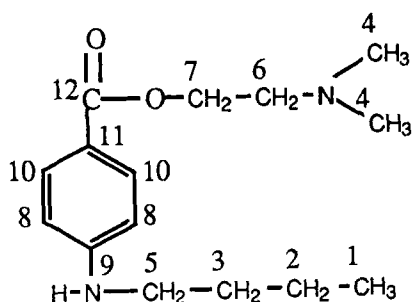


Fig 4 FT-<sup>1</sup>H NMR spectrum

heavy water using DSS as an internal standard are shown in Fig 5 and 6 respectively. The spectral assignments are given in Table 5.



Carbon atom	Chemical shift (ppm)	Off resonance multiplicity
1	15.616	quartet
2	22.094	triplet
3	32.849	triplet
4	44.652	quartet
5	45.506	triplet
6	58.340	triplet
7	60.903	triplet
8	113.631	doublet
9	117.285	singlet
10	134.095	doublet
11	155.738	singlet
12	169.556	singlet

### 5.15 Mass Spectrum

The low resolution electron impact (EI) and chemical ionization (CI) spectra of tetracaine hydrochloride obtained by a double-focusing magnetic sector mass spectrometer VG 70-250-S (VG Analytical, Manchester, England)

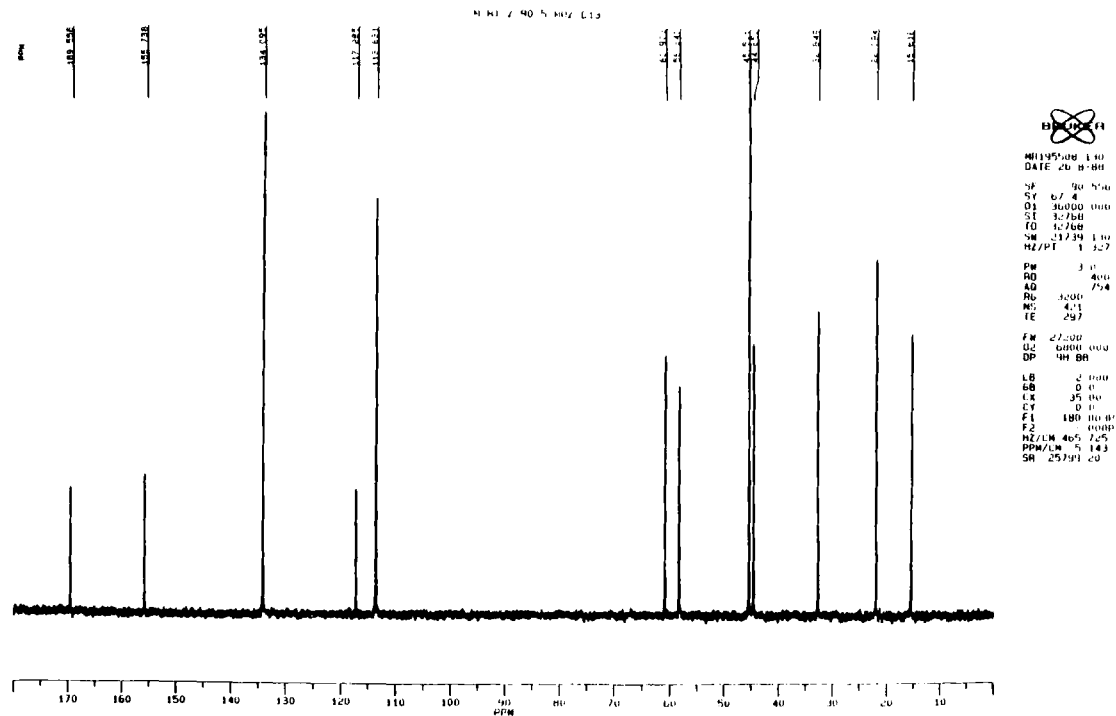


Fig 5 Proton decoupled FT- $^{13}\text{C}$  NMR spectrum

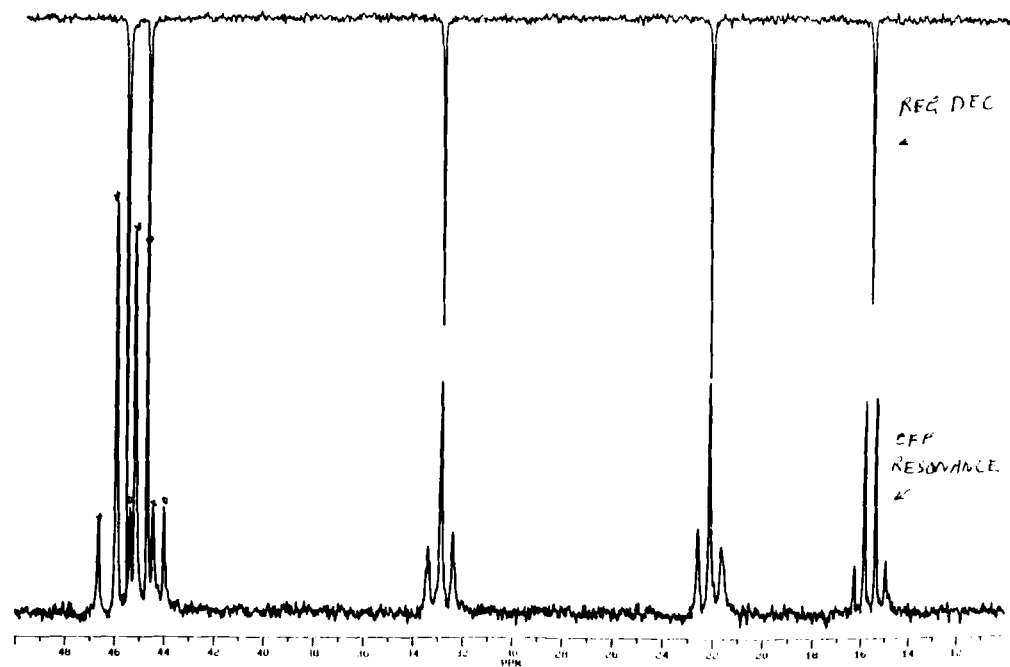


Fig 6 Off resonance proton decoupled FT- $^{13}\text{C}$  NMR spectrum

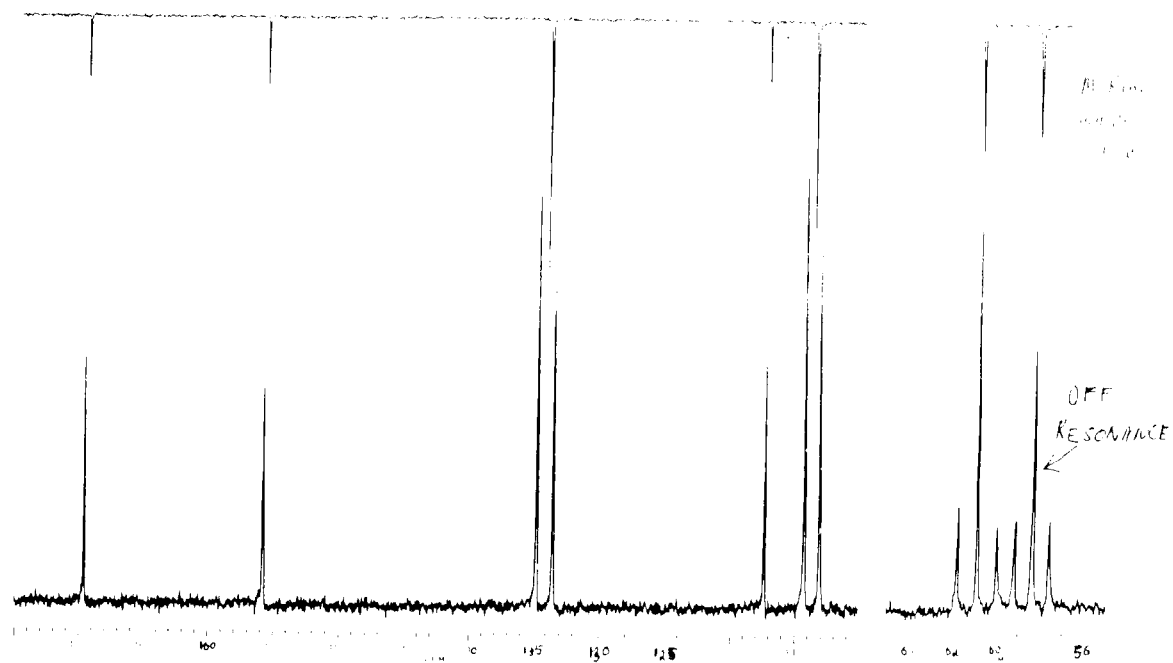


Fig 6 (continued)



are shown in Fig 7-9. The following experimental conditions were used.

#### Electron Impact

Sample introduction: Direct probe

Electron energy: 70 electron volt

Sample temperature: 50 °C

Ion source temperature: 200 °C

#### Chemical ionization

Sample introduction: Direct probe

Electron energy: 200 electron volt

Reagent gas: (i) isobutane

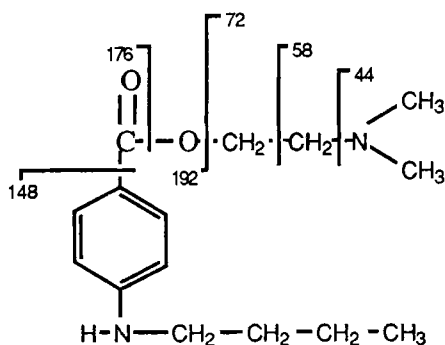
(ii) ammonia

Pressure in ion source region:  $10^{-4}$  mbar

Sample temperature: 100 °C

Ion source temperature: 200 °C

The molecular ion peak at 264 m/e is absent in electron impact mass spectrum(See Fig 7). But protonated molecular ion peak(M+1 or M+H) at 265 m/e is the base peak in chemical ionization mass spectra(See Fig 8 & 9). The assignment of m/e values to positive ions is given in Table 6



m/e	Ion
265	M + 1
266	M + 2
194	COOH <sub>6</sub> H <sub>4</sub> <sup>+</sup> NH <sub>2</sub> C <sub>4</sub> H <sub>9</sub> [·CH <sub>2</sub> CH <sub>2</sub> N(CH <sub>3</sub> ) <sub>2</sub> is lost]

193	$^+ \text{OCOHC}_6\text{H}_4\text{NHC}_4\text{H}_9$
176	$^+ \text{OCC}_6\text{H}_4\text{NHC}_4\text{H}_9$
150	$\text{C}_6\text{H}_5^+ \text{NH}_2\text{C}_4\text{H}_9$
72	$\text{CH}_2=^+ \text{CH}_2-\text{N}(\text{CH}_3)_2$
71	$\cdot \text{CH}_2-\text{CH}=^+ \text{N}(\text{CH}_3)_2$
58	$\text{H}_2\text{C}=^+ \text{N}(\text{CH}_3)_2$
44	$\text{CH}_3-\text{N}=^+ \text{CH}_3$
42	$\cdot \text{CH}=^+ \text{CH}_2-\text{CH}_3$

---

### 5.16 Refractive Index(10)

Refractive indices of two polymeric forms are described in Table 7.

Polymeric Form (m. pt.)	Refractive Indices	
	$\alpha$	$\gamma$
130-136 °C	1.497	> 1.740
139 °C	1.584	1.697

---

## 6. Synthesis

Tetracaine hydrochloride precipitates when hydrogen chloride is passed through a solution of tetracaine in benzene(2). For details about the synthesis of tetracaine see elsewhere(e.g.,11).

## 7. Methods of Analysis

### 7.1 Tests of Identification(5)

Four identification tests have been described.

### 7.2 Titrimetric Methods

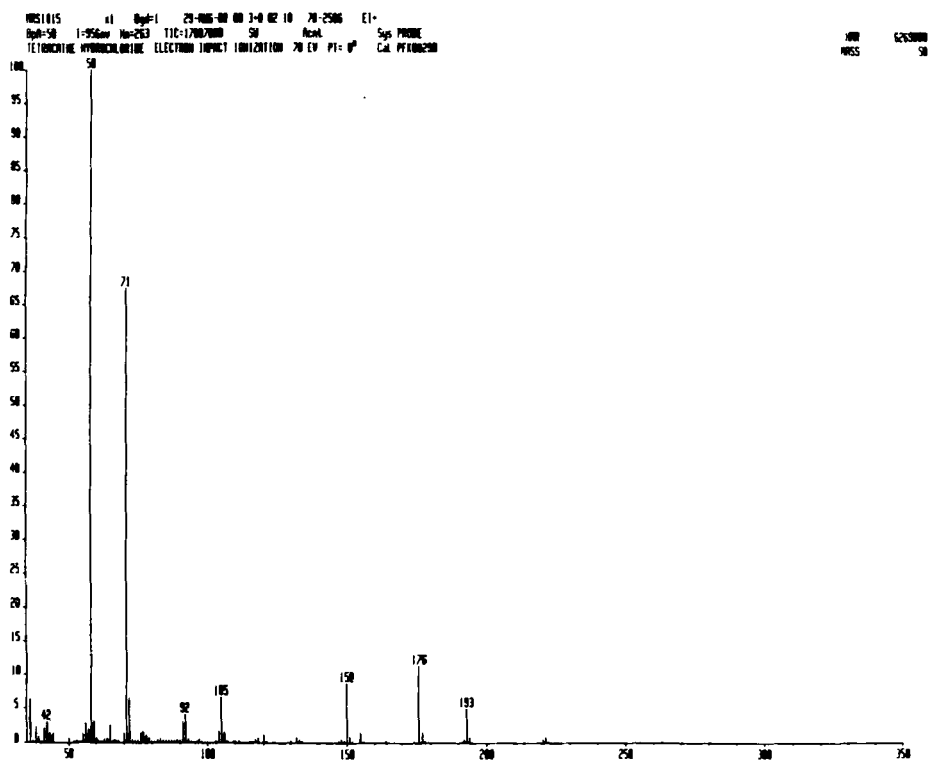


Fig 7 EI mass spectrum

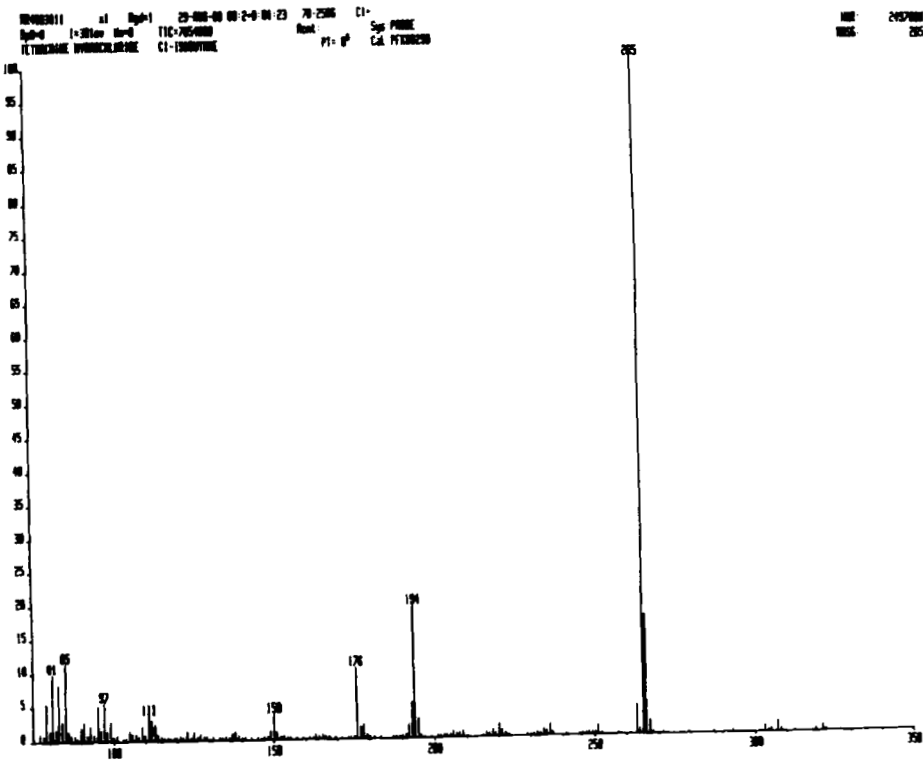


Fig 8 C1-isobutane mass spectrum

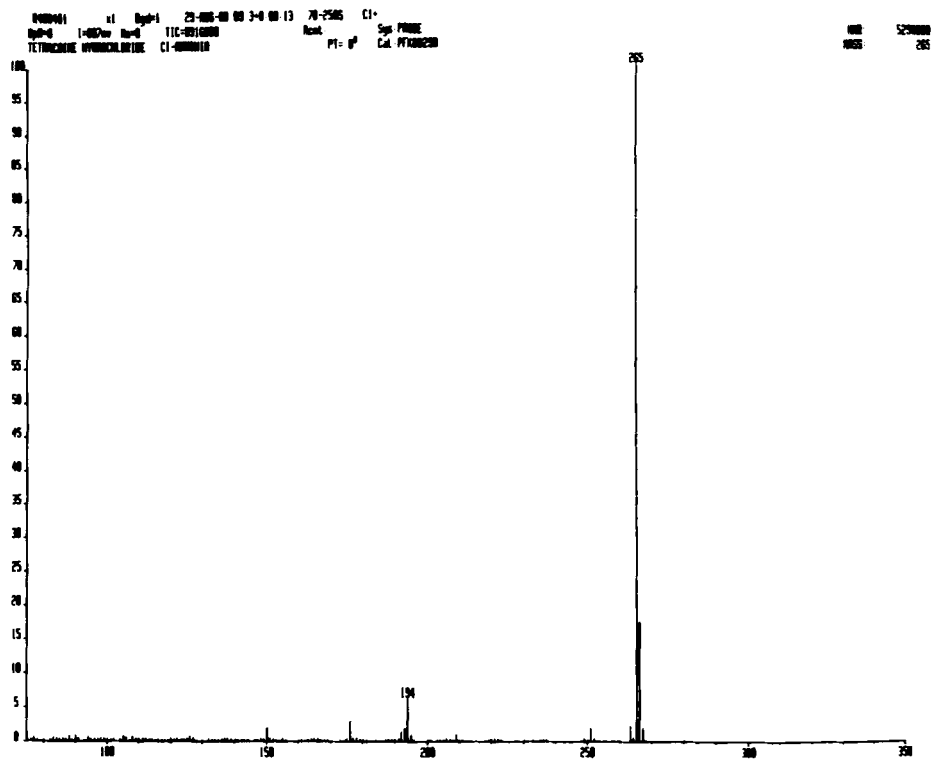


Fig 9 Cl-ammonia mass spectrum

### 7.2.1 Titration with Sodium Nitrite

U. S. P. XXI describes a method for the estimation of tetracaine hydrochloride using 0.1 M sodium nitrite as a titrant. The end-point is determined electrometrically using two platinum electrodes or a platinum and a calomel electrode(12).

### 7.2.2 Spectrophotometric titration with Sodium Nitrite(13)

Dissolve 30 mg of tetracaine hydrochloride in 6 N hydrochloric acid(150 ml) and titrate spectrophotometrically at 385 nm with 0.1 M sodium nitrite. The end-point is marked by a change of slope in a graph of absorbance against volume of the titrant added. The precision is better than 0.8 %. The method has been applied to the determination of tetracaine hydrochloride in the presence of 3:4-dihydroxynorephedrine and a bisulphite compound.

### 7.2.3 Non-aqueous titration with Perchloric Acid

The British Pharmacopoeia(5) describes a method of the estimation of tetracaine hydrochloride using 0.1 M perchloric acid as a titrant and crystal violet as an indicator.

Surmann et al. described the titration of the drug in acetic anhydride medium with perchloric acid using naphtholbenzein as an indicator(14).

### 7.2.4 Non-aqueous titration with Methanesulphonic Acid

Dissolve about 0.1 g of tetracaine hydrochloride in 10 ml anhydrous acetic acid-acetic anhydride(3:1). Add 5 ml of 3 % Hg(II) acetate solution in acetic acid and titrate against 0.1 N methanesulphonic acid in the presence of crystal violet as an indicator. The errors were  $< \pm 0.33$  % for five determinations(15).

### 7.2.5 Non-aqueous conductimetric titration with Perchloric Acid(16).

The conductimetric titration is performed in anhydrous acetic acid(non-aqueous medium) using 0.2 N perchloric acid as a titrant in the presence of mercuric acetate

The end-point is determined graphically by means of a C-24 instrument with platinum electrodes. The errors for the determination were  $< \pm 0.62 \%$ .

### 7.2.6 Potassium Bromate titration(17)

Thirty to fifty mg of the drug are dissolved in 30 ml of water and after the addition of 1.0 g potassium bromide, 3 ml of 30 % nitric acid and one drop of 4-ethoxychrysoidine solution, the mixture is titrated with 0.1 N potassium bromate to a color change from red to yellow. Alternatively the end-point can be determined by an amperometric method.

### 7.2.7 Titration with Arenesulphonic Acid(18)

Five mg of tetracaine hydrochloride are dissolved in 5 ml of water. The solution is adjusted to pH 8 to 9 with saturated aqueous sodium hydrogen carbonate and is extracted with chloroform. The combined extract is filtered and the filtrate is titrated with 0.008 N solution of various arenesulphonic acids with dimethyl yellow as an indicator. The smallest error (-0.58 %) was obtained when 6-hydroxy-3,4-xylene sulphonic acid was used as a titrant.

### 7.2.8 Titration with Cerium(IV) Sulphate(19)

The titrimetric determination of some drugs including tetracaine hydrochloride has been described using cerium(IV) sulphate as an oxidant.

### 7.2.9 Titration with Sodium Dodecyl Sulphate(20)

The determination of tetracaine hydrochloride has been described using 0.01 M sodium dodecyl

sulphate titrant in pH 9.5 borate buffer. The indicator was Azure A.

### 7.2.10 Titration with Hydrochloric Acid

Dissolve about 0.1 g of the salt in 90 % ethanol(10 ml) with warming if necessary, and pass the solution at 2 to 3 ml per minute through an anion-exchange resin(Merck III). Pass 90 % ethanol(10 ml) through the column at the same rate, then rapidly pass a further 20 ml of hot ethanol. Dilute the combined percolates with freshly boiled cooled water(50 to 70 ml) and titrate the liberated amine(tetracaine) with 0.1 N hydrochloric acid in the presence of Tashiro's indicator(21).

The British Pharmacopoeia(22) describes a method for the determination of tetracaine hydrochloride. The method involves extraction of the drug with ether in sodium carbonate solution, the addition of 10 ml of 0.1 N hydrochloric acid. The reaction mixture is titrated against 0.1 N sodium hydroxide. Each ml of 0.1 N hydrochloric acid is equivalent to 0.03008 g of tetracaine hydrochloride.

### 7.2.11 Coulometric Titration(23)

The following were the reaction conditions during the determination of tetracaine hydrochloride.

Titrant:  $(C_6H_5)_4 BH/Ag^+$   
Supporting electrolyte: 0.4 M  
 $NaNO_3$  in  
acetone

Working electrode: Ag  
Sample size: 10-20 mg  
Detection: Potentiometric, Pt  
indicator vs  
Ag/AgCl  
reference  
Precision: 0.2 % relative standard  
deviation



### **7.3 Spectrophotometric Methods**

#### **7.3.1 Colorimetric Methods**

Tetracaine hydrochloride in aqueous solution can be determined by Bratton-Marshall reagent. A colored reaction takes place and absorbance is noted at 500 nm. From a calibration curve, the concentration of unknown solution is determined(24).

A colorimetric method for the determination of nonhydrolyzed tetracaine in water, plasma, spinal fluid is based on the selective extraction of the coloured salt formed between tetracaine and bromothymol blue. Absorbance is noted at 410 nm(25).

El-Kommos and Sidhom(26) described a method for the quantitative determination of tetracaine hydrochloride in various dosage forms. It is based on the interaction of tetracaine hydrochloride with p-dimethylaminocinnamic aldehyde in acidic medium to form a stable pink-colored product with absorption maximum at 510 nm. Beer's law was obeyed over the range 0.5-20 µg/ml. The colored complex was produced within 5 minutes and was stable for at least 12 hours.

#### **7.3.2 Ultraviolet Methods**

U. S. P. XXI (12) describes ultraviolet method for the determination of tetracaine hydrochloride in various dosage forms. Absorbance is noted at 310 nm. The amount of tetracaine hydrochloride in each dosage form is determined by a specific formula.

A method for the determination of tetracaine hydrochloride in cartridges used for dental anesthesia has been described. Mix a 50 ml aliquot with 7 % sodium nitrite solution(1 ml) and one ml of 1 N HCl . After 5 min., add 10 % sodium carbonate solution(2 ml) and 1 % sodium 8-amino-1-naphthol-2:4-disulphonate solution(2 ml). Set aside for 1 min.

and extract with chloroform(3 x 25 ml). Wash the combined extracts with water(15 ml), filter through paper, dilute with chloroform to 100ml and measure the extinction at 290 nm against a blank. The method is also applicable to a sample which contains both tetracaine hydrochloride and procaine hydrochloride(27).

Porush et al. (28) described a method for the determination of intact tetracaine from blood in humans : Blood is withdrawn from the cubital vein and oxalated in a test tube in the usual manner. The drug is extracted with chloroform and absorbance of the organic phase is noted at 303 nm.

#### 7.4 Polarographic Method(29)

To a solution, 0.4-0.8 ml containing 0.5-1.0 mg of tetracaine hydrochloride, add 5 ml of 1 N hydrochloric acid and 2 ml of 1 N sodium nitrite. Mix and let stand for 5 min. Add 3 ml of 20 % potassium hydroxide and 0.2 ml of 0.2 % gelatin solution, and then dilute with to 15 ml. Determine the current height at -1.7 volt and calculate tetracaine hydrochloride in the sample from a prepared standard curve(current height vs concentration of drug).

#### 7.5 Chromatographic Methods

##### 7.5.1 Column Chromatography

Doyle and Proctor(30) separated mixtures of local anesthetics including those containing tetracaine hydrochloride by the ion-pairing column chromatographic method. The percentage recovery for a mixture of procaine hydrochloride and tetracaine hydrochloride was  $99.3 \pm 1.6$  and  $98.9 \pm 3.82$  % respectively.

##### 7.5.2 Gas Liquid Chromatography

The following chromatographic conditions were used in system A & B.

System A (31)

Column: Column (6ft x 4 mm) packed with 3.8 %  
silicone rubber UC W 98 on Diatoport S,  
80/100 mesh (Hewlett Packard).

Column temperature: 200 °C

Detector temperature: 230 °C

Injection port temperature: 200 °C

Attenuation: 64

Carrier gas(nitrogen)flow rate: 50 cc/min

Hydrogen flow rate: 35 cc/min

Air flow rate: 300 cc/min

Detector: Flame ionization

Internal standard(I.S.): Procaine  
hydrochloride(10  
mg/ml in water)

Retention time: 11 min. (tetracaine  
hydrochloride), 5  
min.(procaine hydrochloride)

Sample: Inject 3 µl chloroform extract

The concentration of tetracaine hydrochloride was determined by the following formula.

Concentration(mg/ml) =(Area of sample peak/Area of  
internal standard peak) X (Area of I.S. peak/Area of R.S. peak) x  
(Conc. of R. S. in mg/ Volume in ml of sample used)

where R. S. is reference standard. It was  
tetracaine hydrochloride(10 mg/ml in water).

System B(32)

Column: Glass column(5 ft x 0.25 inch) packed  
with 2.5 % OV-1 silicone on acid-  
washed and silanised chromasorb-G

Column temperature: 225 °C

Detector (flame ionization)temperature:

290 °C

Injection port temperature: 265 °C

Attenuation : 20 or 50 x 10<sup>2</sup>

Chart speed: 120 nm per hour  
Carrier gas: Nitrogen  
Carrier gas flow rate: 50 ml/min  
Internal Standard: Butacaine sulphate  
Sample: Inject 8  $\mu$ l of ether extract

A calibration curve of tetracaine/butacaine peak ratio against tetracaine hydrochloride concentration (10 to 50 mg) was made. The method was used for the determination of tetracaine hydrochloride in eye drop preparations.

### 7.5.3 High Performance Liquid Chromatography(33)

Mennon and Norris described a simple HPLC method for the simultaneous determination of tetracaine hydrochloride and its hydrolytic degradation product, p-n-butylaminobenzoic acid in tetracaine hydrochloride injection. The following chromatographic conditions were used.

Column: 30 cm x 4 mm column containing microparticulate (10  $\mu$ m) bonded to octadecylsilane.  
Eluent: Water-acetonitrile-methanol (60:20:20) containing 0.06 % (v/v) sulphuric acid, 0.5 % w/v sodium sulfate and 0.02 % w/v sodium heptanesulfonate (pH 2.6)  
Internal standard solutions: (1) A 10 mg/ml salicylic acid solution in methanol-water (1:1) for tetracaine hydrochloride. (2) A 10 mg/ml of propiophenone in methanol-water (1:1) was used as the internal standard for p-n-butylaminobenzoic acid.  
UV detector response at 305 nm: (1) Linear for tetracaine hydrochloride in the 0.4-2.0 mg/ml range. (2) Linear for p-n-butylaminobenzoic acid in the range 0.003-0.02 mg/ml.

### 7.5.4 Paper Chromatography(34)

The following experimental conditions were used.

Solvent system: n-butanol-acetic acid-  
water(5:1:3)  
Detection: Modified Dragendorff's reagent  
Development: Descending  
Sample size: 5-25 µg tetracaine  
hydrochloride  
Paper: Whatman 1  
R<sub>F</sub> value: 0.83

The quantitative analysis of the colored spots was done by photoelectric densitometry.

### 7.5.5 Paper Electrophoresis(35)

The paper electrophoresis of some local anesthetics including tetracaine hydrochloride was carried out on a Whatman paper No. 4 at 20 V per cm in Britton-Robinson buffer solution with 1 µl of the sample(about 0.005 to 0.008 mg). Dragendorff's reagent was used for the detection of the separated compounds.

### 7.6 Capillary Isochophoresis(36)

An analytical method for the simultaneous qualitative and quantitative determination of lidocaine hydrochloride, procaine hydrochloride and tetracaine hydrochloride from pharmaceutical dosage forms utilising isochophoresis technique has been reported. The separation occurs due to the difference in the mobilities of various local anesthetics. The recovery rates of 97 % were obtained by this method without interference from other ingredients in the formulations.

### 7.7 Refractive Index Method(37)

A calibration curve was constructed between refractive index and tetracaine hydrochloride concentration. The medium used was 0.002 % phenylmercuric nitrate containing 0.1 % sodium metabisulphite.

### 7.8 Gravimetric Method(29)

Transfer to a separatory funnel about 0.3 g of tetracaine hydrochloride, accurately weighed, add 25 ml of water, make alkaline with 4 % sodium hydroxide solution and extract with seven 20 ml portions of ether. Wash the combined ether extracts with 15 ml of water, filter the ether solution through cotton and wash the vessel and the filtrate with two 10 ml portions of ether. Evaporate the combined ether solutions to a thick oil-like consistency with the aid of a current of warm air, and dry over silica gel to a constant weight. The weight of the residue multiplied by 1.138 represents the weight of tetracaine hydrochloride.

### 7.9 Ion Selective Electrode Method(38)

The drug was determined by using a chloride ion selective electrode.

## 8. Stability

Tetracaine hydrochloride powder should be stored in air tight containers, protected from light(3). The preparations should be stored at 2-8 °C and also be protected from light (39). In aqueous solution, tetracaine hydrochloride hydrolyzes to n-butylaminobenzoic acid and 2-dimethylaminoethanol; decarboxylation of n-butylaminobenzoic acid to butylaniline occurs and the butylaniline oxidizes to form various colored products. The hydrolysis of the drug is catalysed by both hydrogen and hydroxyl ions. At 25 °C, solutions are most stable at about pH 3.5(3). The degradation product, p-n-butylaminobenzoic acid is only sparingly soluble in water and potentially can cause crystal deposition in amethocaine hydrochloride preparations such as injections, eye drops, topical solution and sterile solution(39).

The addition of alkali hydroxides or carbonates to tetracaine hydrochloride solutions precipitates tetracaine base as an oily liquid(39). The stability of the drug appear to be unaffected by the addition of dextrose(40). The stability of

amethocaine hydrochloride solutions in the presence of various surfactants has been reported(41).

#### 9. Pharmacokinetics(39)

The drug has a delayed onset of action, often up to 15 min. in large nerve trunks. The duration of action during spinal anesthesia is about 1.5 to 3 hours. It is hydrolyzed to p-aminobenzoid acid by plasma pseudocholinesterase. The metabolites are excreted mainly by the kidneys but in animals, biliary excretion of tetracaine also occurs.

#### 10. Toxicity(42)

LD <sub>50</sub> (intravenous in mice)	13 mg/Kg
LD <sub>50</sub> (subcutaneous in mice)	35 mg/Kg

#### 11. Dosage Forms

##### (1) U.S.P. XXI(12)

Ophthalmic solution, solution, ointment, ophthalmic ointment, cream, injection, topical solution, sterile.

##### (2) British Pharmacopoeia (5)

Eye drops

#### 12. Uses(43)

As a local and spinal anesthetic.

#### 14. References

- (1) The Merck Index, 10th Edn., p 1314(No. 9014), Merck & Co., Rathway, New Jersey, (1983).
- (2) Remington's Pharmaceutical Sciences, 17th Edn., p1054 Mack Publishing Co., Pennsylvania(1985).
- (3) The Pharmaceutical Codex, 11th Edn., p 26, The Pharmaceutical Press, London(1979).

- (4) *Atlas of Spectral Data and Physical Constants for Organic Compounds*, 2nd Edn., Vol. 4, p 581, CRC Press, Ohio(1975).
- (5) *British Pharmacopoeia*, pp 5-6, Her Majesty's Stationary Office, London(1980, addendum 1982).
- (6) *Clarke's Isolation and Identification of Drugs*, 2nd Edn., pp 335-336, The Pharmaceutical Press, London(1986).
- (7) F. A. Vilallonga and E. W. Phillips, *J. Pharm. Sci.*, 68, 314 (1979).
- (8) D. Attwood and P. Fletcher, *J. Pharm. Pharmacol.*, 38, 494 (1986).
- (9) R. C. Sullivan and K. P. O'Brien, *Bull. Narcotics*, 20, 31 (1968).
- (10) A. Wickstorm, *J. Pharm. Pharmacol.*, 5, 158 (1953)
- (11) O. Eisler, U. S. Patent 1,889,645 (1932); British Patent 815,144 (Abbot, 1959); I. Kh. Fel'dman and E. L. Kopeliovich, *J. Appl. Chem. (USSR)* 17, 588 (1944) through *Chem. Abs.*, 40, 2268 (1946).
- (12) *The United States Pharmacopela*, Vol. XXI, pp 1030-1032, United States Pharmacopeial Convention, Rockville, Madison (1985).
- (13) E. L. Pratt, *J. Am. Pharm. Ass. Sci. Ed.*, 46, 724 (1957).
- (14) P. Surmann, C. W. H. Dietz and T. Nassauer, *Dtsch Apoth.-Ztg*, 123, 1110 (1983) through *Anal. Abs.*, 45, 5E2 (1983).
- (15) H. Ellert and E. Sell, *Farmacja Pol.*, 22, 745 (1966) through *Anal. Abs.*, 15, 1024 (1968).
- (16) H. Ellert and E. Sell, *Farmacja Pol.*, 23, 453 (1967) through *Anal. Abs.*, 15, 4985 (1968).
- (17) G. Bozai-Bolda and L. Mosonyi-Sata, *Gyogyszereszet*, 14, 181 (1970) through *Anal. Abs.*, 20, 3342 (1971).
- (18) Z. Zdzislaw, *Farmacja Pol.*, 26, 819 (1970) through *Anal Abs.*, 21, 4374 (1971).
- (19) I. P. Koka, *Farmatsiya (Moscow)* 29, 49 (1980) through *Anal. Abs.*, 42, 2E4 (1982).
- (20) M. Massaccesi, *Boll. Chim. Farm.*, 120, 297 (1982) through *Anal. Abs.*, 43, 3E56 (1982).



- (21) W. Schneider, Dtsch. ApothZtg., 101, 1259 (1961) through Anal. Abs., 9, 2479 (1962).
- (22) British Pharmacopoeia, p 33, The Pharmaceutical Press, London (1968).
- (23) R. E. Schrimmer, Modern Methods of Pharmaceutical Analysis Vol. 2, p 232 CRC Press, Florida (1982); G. J. Patriache and J. J. Lingane, Anal. Chim. Acta, 37, 455 (1967).
- (24) O. Hardy and A. Slouf, Cesk. Farm., 5, 21 (1956) through Chem. Abs., 50, 9946i (1956).
- (25) M. E. Auerbach, D. L. Davis and F. F. Foldes, Federation Proc., 11, 319 (1952)
- (26) M. E. El-Kommos and M. B. Sidhom, Arch. Pharm. Chemi. Sci. Ed., 10, 57 (1982).
- (27) M. E. Auerbach and M. M. Tuckerman, J. Am. Pharm. Ass. Sci. Ed., 48, 194 (1959).
- (28) I. Porush, A. Shimamura, L. T. Takahashi, J. Pharm. Sci., 54, 1809 (1965).
- (29) C. A. Kelly In Encyclopedia of Industrial Chemical Analysis (F. D. Snell and C. L. Hilton Eds.) 2nd Edn. Vol. 5, pp 414-421, Interscience Publishers, New York (1967).
- 30) J. B. Proctor and T. D. Doyle, J. Assoc. Off. Anal Chem., 58, 93 (1975); T. D. Doyle and J. B. Proctor, J. Assoc. Off. Anal. Chem., 58, 88 (1975).
- (31) J. A. Patel, Am. Hosp. Pharm., 27, 1009 (1970)
- (32) N. D. Greenwood, J. Hosp. Pharm., 29, 240 (1971)
- (33) G. N. Mennon and B. J. Norris, J. Pharm. Sci., 70, 569 (1981).
- (34) H. Schrifftman, J. Am. Pharm. Ass. Sci. Ed., 48, 111 (1959).
- (35) V. Jokl and Sukupova-Kolkovous, Ceskosl. Farm., 10, 197 (1961) through Anal. Abs., 8, 4338 (1961).
- (36) V. Klein, Arzneim Forsch, 32, 795 (1982).
- (37) R. N. Longshaw and N. Walli, J. Hosp. Pharm., 31, 36 (1973).
- (38) P. Erzebet, P. Erno, P. Magy, Kem. Foly, 76, 573 (1970) through Anal. Abs., 21, 2900 (1971).
- (39) Drug Information 88, p 1857, American Society of Hospital Pharmacists, Madison (1988).
- (40) N. Harb, J. Hosp. Pharm., 26, 44 (1969).

- (41) A. I. Tentsova, L. P. Frik, T. V. Izhendeeva and M. E. Pudel, *Farmatitsiya* (Moscow) 35, 22 (1986).
- (42) B. A. Bopp, A. T. Dren, D. E. Ebert and A. O. Geiszler, *J. Pharm. Sci.*, 67, 882 (1978).
- (43) *Americal Drug Index*, 32nd Edn, p 592, J. B. Lippincott, Co., Philadelphia (1988).

*Analytical Profile of Thiamine Hydrochloride*

*By*

*Khalid Abdul Mohsen Al-Rashood*

*Fahad Jaber Al-Shammery\**

*Neelofur Abdul Aziz Mian\**

*Pharmaceutical Chemistry Department, College of Pharmacy,  
King Saud University, Riyadh, Saudi Arabia.*

*\*Clinical Laboratories Department, College of Applied  
Medical Sciences, King Saud University, Riyadh,  
Saudi Arabia.*

## Contents

1. Description
  - 1.1 Nomenclature
  - 1.2 Formulae
  - 1.3 Molecular Weight
  - 1.4 Elemental Composition
  - 1.5 Appearance, Color, Odor and Taste
  - 1.6 Occurance.
2. Physical Properties
  - 2.1 Melting Range
  - 2.2 Solubility
  - 2.3 pH and Stability
  - 2.4 Spectral Properties
3. Synthesis
4. Metabolism
5. Vitamin Deficiency
6. Human Requirements
7. Methods of Analysis
  - 7.1 Elemental Analysis
  - 7.2 Identification
  - 7.3 Titrimetric Method
  - 7.4 Gravimetric Method
  - 7.5 Volumetric Method
  - 7.6 Colorimetric Method
  - 7.7 Spectrophotometric Method
  - 7.8 Polarographic Method
  - 7.9 Fluorimetric determination
  - 7.10 Atomic Absorption Spectroscopy
  - 7.11 Chromatographic Methods
8. Acknowledgement
9. References

## Thiamine Hydrochloride

### 1. Description

#### 1.1 Nomenclature

##### 1.1.1 Chemical Names

a) 3-[(4-Amino-2-methyl-5-pyrimidinyl)-methyl]-5-(2-hydroxyethyl)-4-methylthiazolium chloride monohydrochloride. (1)

b) 3-(4-Amino-2-methylpyrimidin-5-methyl)-5-2-hydroxyethyl)-4-methylthiazolium chloride hydrochloride. (2)

##### 1.1.2 Generic Names

Thiamine Hydrochloride, Vitamin B<sub>1</sub>.

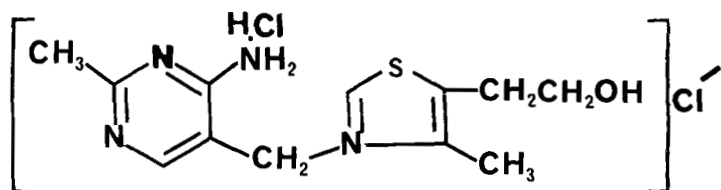
##### 1.1.3 Trade Names

Vitamin B Hydrochloride, Thiamine chloride-hydrochloride, Aneurine hydrochloride, Thiaminium-chloride Hydrochloride, Bedome, Belgiolan, Benerva, Bequin, Betabion hydrochloride, Betalin S, Betraxin, Bethiazine, Bevitex, Bewon, Biuno, Bivatin, Bivita, Clotiamina, Metabolin, Thiadoxine, Thiavit, Tiamidon, Tiaminal, Vitaneuron, Thiaminii chloridum, Thiamini Hydrochloride, Aneurine chloride Hydrochloride, Thiaminium chloride, Thiamin Hydrochloride, Thiamine chloride.

### 1.2 Formulae

#### 1.2.1 Empirical

C<sub>12</sub>H<sub>18</sub>Cl<sub>2</sub>N<sub>4</sub>OS.

1.2.2 Structural1.2.3 CAS Registry Number

59-43-8

1.2.4 Wiswesser Line Notation

T6N CNJ B DZ E1-AT5K CSJ  
D2Q E & G & GH.

1.3 Molecular Weight

337.25

1.4 Elemental Composition

C 42.73%	H 5.38%	Cl 21.03%	N 16.61%
O 4.74%	S 9.51%		

1.5 Appearance, Color, Odour and Taste

Colourless crystals or white crystalline powder with a characteristic somewhat meat-like odour and a bitter taste. (2)

1.6 Occurance

Occurs in plants and in animal tissues, notably in rice husk, cereal grains, yeast, liver, eggs, milk, green leaves, roots and rice bran. Practically all vitamin B<sub>1</sub> sold is synthetic (1).

## 2. Physical Properties

### 2.1 Melting Range

248° with decomposition. (1)

### 2.2 Solubility

One gram dissolves in about 1 ml of water, 18 ml glycerol, 100 ml 95% alcohol, 315 ml abs. alcohol, more soluble in methanol soluble, in propylene glycol. Practically insoluble in ether, benzene, hexane, chloroform.

### 2.3 pH and Stability

pH of a 1% w/v solution in water is 3.13, pH of a 0.1% w/v solution in water is 3.58. A 2.5% solution in water has a pH of 2.7-3.3. A 4.24% w/v solution is iso-osmotic with serum. (2) Caused 87% haemolysis of erythrocytes cultured in it for 45 minutes. The solution and erythrocytes darkened in colour. (2) On exposure to air of average humidity, the vitamin absorbs an amount of water corresponding to nearly one mol, forming a hydrate. In the dry form the vitamin is stable and heating at 100° for 24 hrs does not diminish its potency. In aq. solution it can be sterilized at 110° but if the pH of the solution is above 5.5 it is destroyed rapidly. (1).

Sterile solutions of pH 4 or less lose activity only very slowly but neutral or alkaline solutions deteriorate rapidly, especially in contact with air. When exposed to air, the anhydrous material rapidly absorbs about 4% of water (2).

Thiamine hydrochloride injection is a sterile solution of the drug in water for injection, the pH of injection should be maintained from 2.5-4.5 (4).

Accelerated stability testing at 40°C and 70°C, with 60% relative humidity, suggested that tablets prepared by benzoyl thiamine disulphide were more stable than those prepared with thiamine hydrochloride or mononitrate. (2).

The stability of thiamine was at its maximum at pH 2 and decreased with increase in pH. In water the stability after 6 months storage at pH 2 and 37° was 100%.

## 2.4 Spectral Properties

### 2.4.1 Ultraviolet Spectrum

The UV spectrum of thiamine hydrochloride in water (Fig. 1) was scanned from 200 to 340 nm using LKB ultrospec plus UV/visible spectrophotometer. It exhibited a  $\lambda_{\max}$  at 232 nm. (2a)

### 2.4.2 Infrared Spectrum

The I.R. spectrum of thiamine hydrochloride (Fig. 2) was recorded on a Perkin Elmer-580 B Infrared Spectro-photometer to which an infrared data station is attached. The structural assignments are mentioned in Table (1).

Table (1). IR characteristics of thiamine hydrochloride

<u>Frequency <math>\text{cm}^{-1}</math></u>	<u>Assignment</u>
3420, 3495	-NH <sub>2</sub>
3255	-OH
3000	Aromatic -CH
2930	Aliphatic -CH
1650	C = N
1600	Aromatic C = C

The other infrared data for thiamine hydrochloride are reported (2b) and are as follows:

3290, 2940, 2700, 1670, 1610, 1520, 1390, 1250, 1220, 1040, 890, 830, 830, 770, 750, 700.



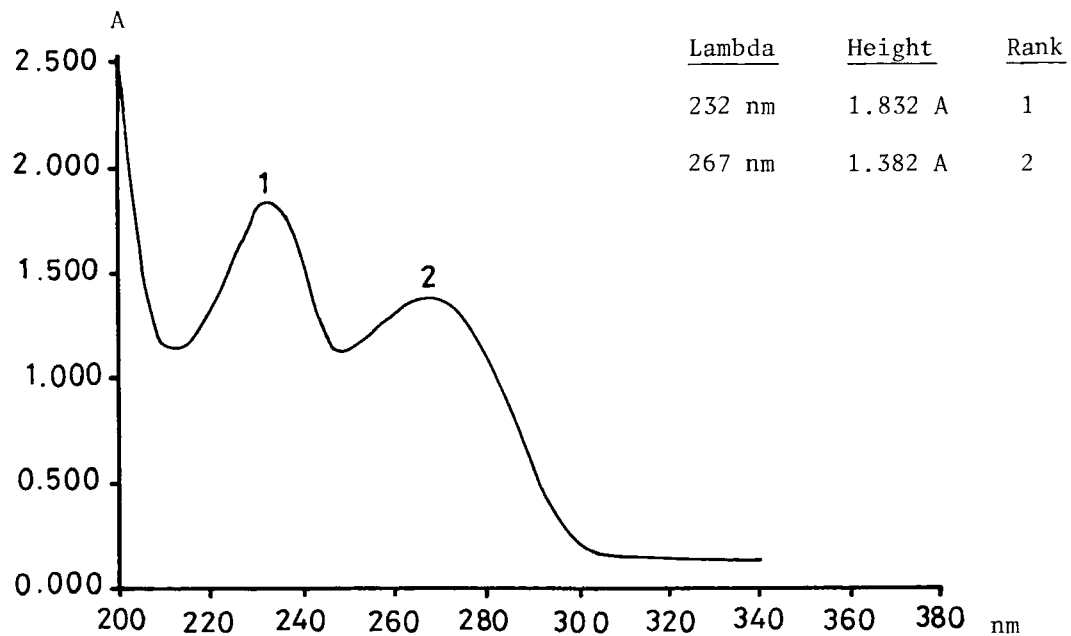


Fig. 1: U.V. Spectrum of Thiamine Hydrochloride in  $H_2O$ .

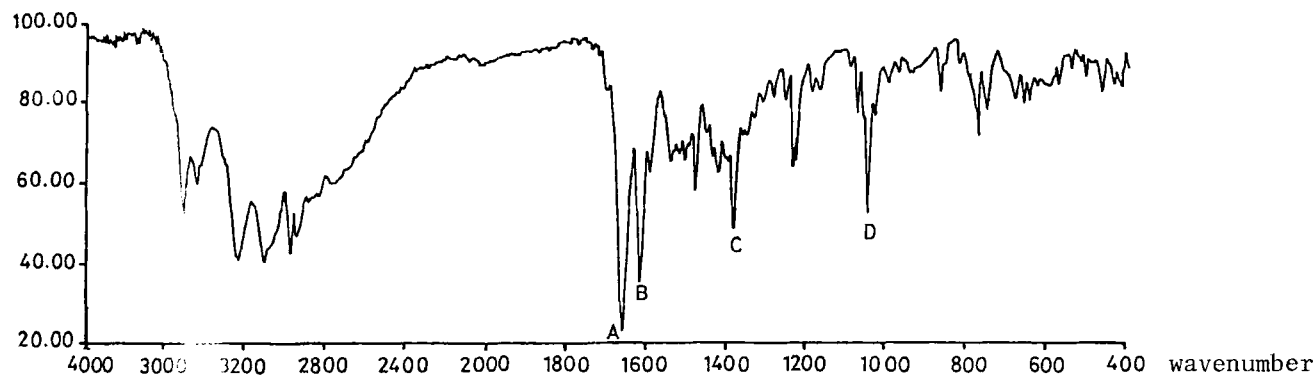


Fig. 2: I.R. Spectrum of Thiamine Hydrochloride as KBr disc.

### 2.4.3 Proton Spectrum

The PMR spectrum of thiamine hydrochloride in D<sub>2</sub>O (Fig. 3) was recorded on a Varian FT 80 NMR spectrophotometer (2a). The following structural assignments have been made (Table 2).

Table (2). PMR characteristics of thiamine HCl

<u>Group</u>	<u>Chemical shift <math>\delta</math> (ppm)</u>
-CH <sub>3</sub>	2.53 s
-CH <sub>3</sub>	2.61 s
-CH <sub>2</sub>	3.17 d
-CH <sub>2</sub>	3.85 m
N-CH <sub>2</sub>	5.55 s

---

s = singlet, d = doublet, m = multiplet

Other reported data in D<sub>2</sub>O are 2.6, 2.7, 3.3, 4.0, 5.7, 8.1 , 9.8.

### 2.4.3.2 <sup>13</sup>C-NMR Spectra

The <sup>13</sup>C NMR spectrum of thiamine hydrochloride in D<sub>2</sub>O using TMS as an internal reference is recorded on a Varian XL 200 MHz NMR spectrophotometer (2a) and is presented in Fig. (4).

### 2.4.4 Mass Spectrum (2a)

The mass spectrum of thiamine obtained by electron impact ionization (EI) is shown in Fig. (5). The mass spectral data are shown in Table (3).

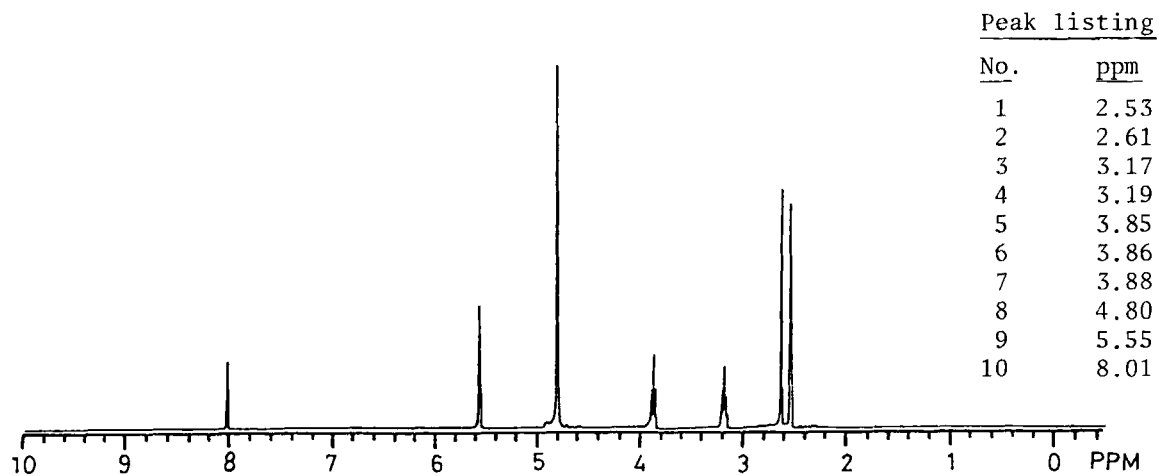


Fig. 3: PMR Spectrum of Thiamine Hydrochloride in  $D_2O$ .

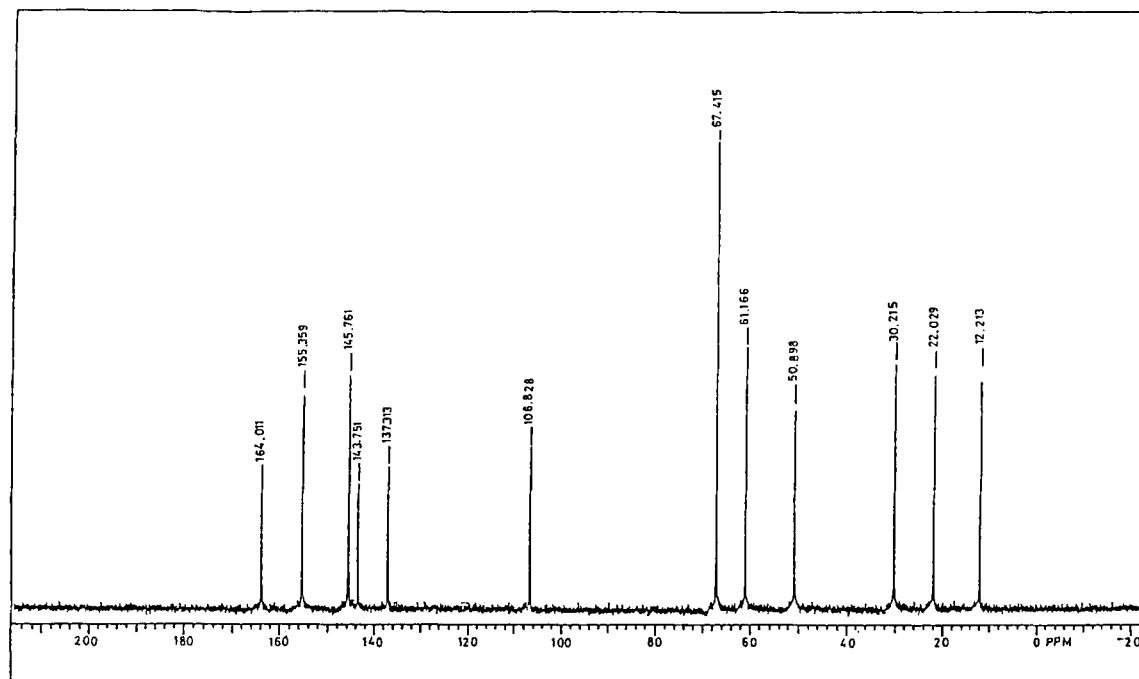


Fig. 4:  $^{13}\text{C}$ -NMR Spectrum of Thiamine Hydrochloride in  $\text{D}_2\text{O}$ .

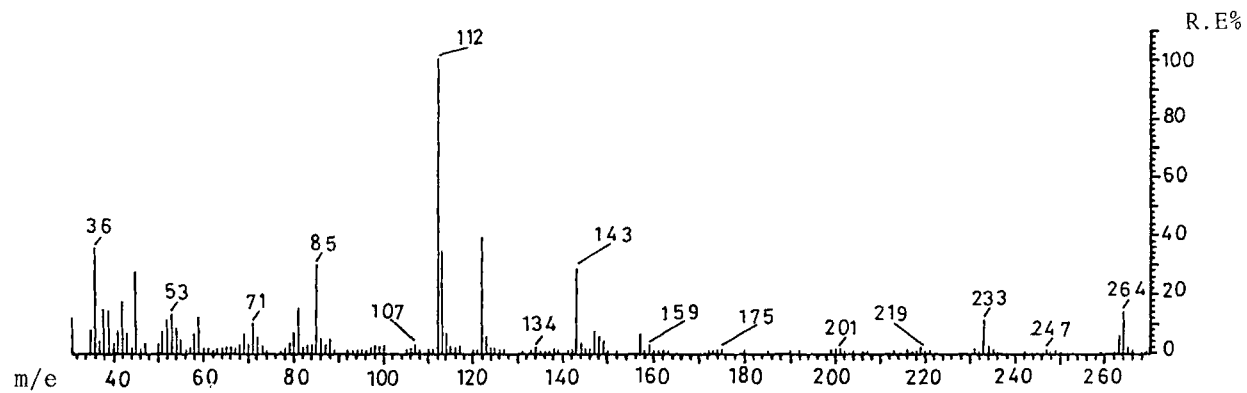
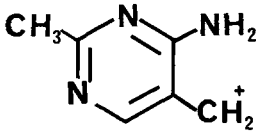
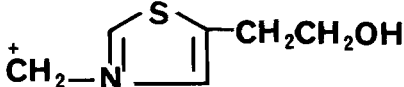
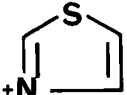


Fig. 5: EI-Mass Spectrum of Thiamine

Table (3). The most prominent fragments of thiamine HCl

<u>m/e</u>	<u>Relative intensity (%)</u>	<u>Fragment</u>
143	30	
122	42	—
112	100	
85	30	
45	26	+CH <sub>2</sub> CH <sub>2</sub> OH
36	34	—

### 3. Synthesis

The synthesis of thiamine has been performed in different ways.

#### Scheme 1 (3)

It is possible to synthesize the pyrimidine nucleus and the thiazole nucleus separately and afterwards to connect both parts. It is also possible to synthesize one of the nuclei with an extra side branch and afterwards to connect both parts.

It is also possible to synthesize one of the nuclei with an extra side branch and afterwards to build up the other ring from the side branch.

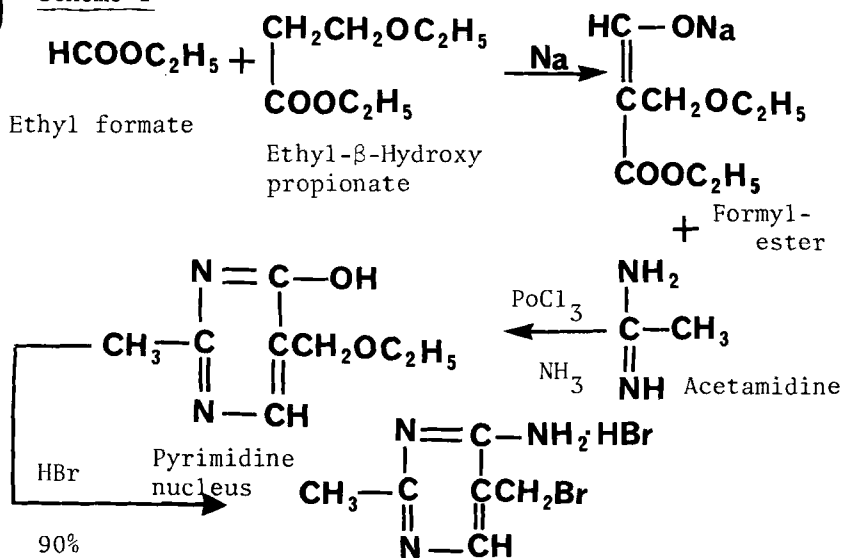
a) For synthesizing the pyrimidine part ethylformate and  $\beta$ -ethoxypropionate may be condensed with Na then the product is condensed with acetamidine. The hydroxyl group is converted into  $\text{NH}_2$  group by treating first part with  $\text{POCl}_3$  and then with  $\text{NH}_3$ . The ethoxy group is converted into a bromide and the product formed is pyrimidine nucleus.

b) The thiazol moiety may be synthesized in several ways. The method of Buchman consists in condensing thioformamide with bromoacetopropanol.

By heating the hydrobromide of the pyrimidine compound with the thiazol compound, the thiamine hydrobromide is formed.

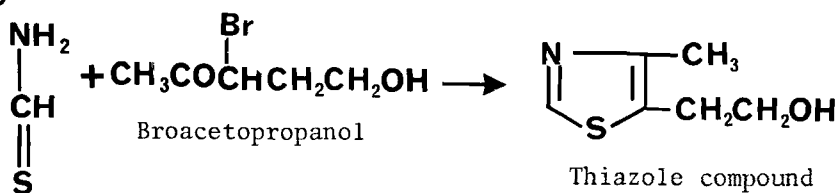
The bromide hydrobromide may be converted into the chloride hydrochloride by treating with  $\text{AgCl}$  or by precipitating the practically insoluble thiamine picrate and dissolving it in hydrochloric acid.

(a) Scheme I

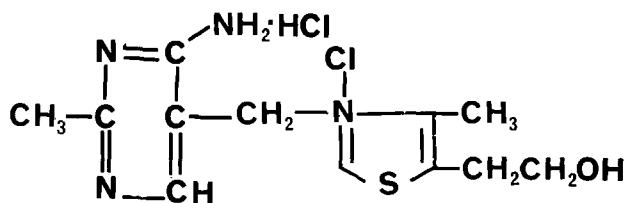
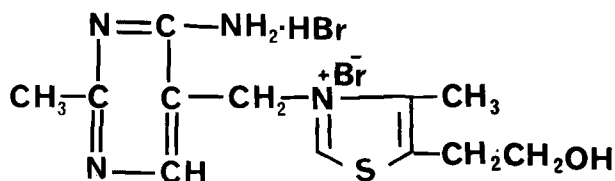
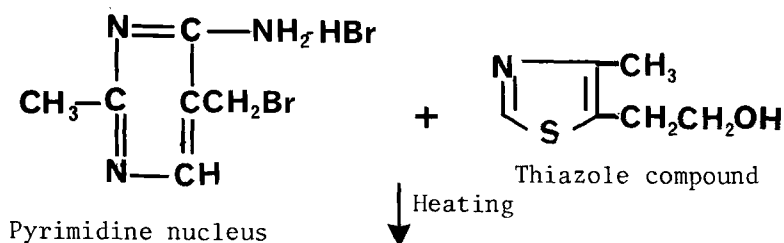




## (b) Scheme I (Continued)



Thioformamide



Thiamine Hydrochloride

Scheme II (4)

This vitamin consists of two ring systems pyrimidine portion and a thiazole portion joined by a methylene bridge.

The pyrimidine may be prepared by several processes one of which is as follows:

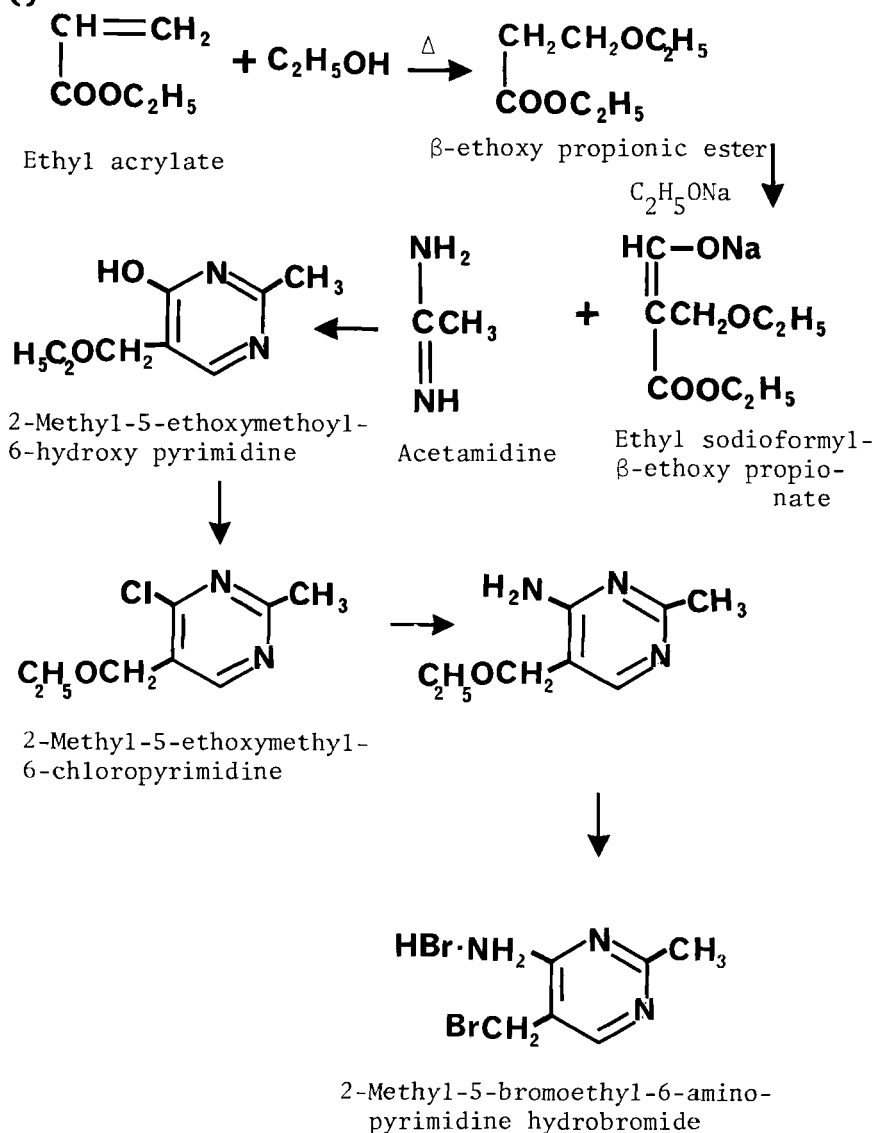
Ethyl acrylate ( $\text{CH}_2 = \text{CHCOOC}_2\text{H}_5$ ) is heated with ethyl alcohol forming  $\beta$ -ethoxypropionic ester [ $\text{C}_2\text{H}_5\text{OCH}_2\text{CH}_2\text{COOC}_2\text{H}_5$ ] which is condensed in the presence of sodium metal with formic acid to form ethyl.sodioformyl- $\beta$ -ethoxypropionate [ $\text{C}_2\text{H}_5\text{OCH}_2\text{CNa}(\text{CHO})\text{COOC}_2\text{H}_5$ ]. This is then condensed with acetamidine yielding 2-methyl-5-ethoxymethyl-6-hydroxypyrimidine. This compound is treated with phosphorous oxychloride thereby replacing the OH on carbon 6 with Cl, and by reaching the resulting chloro derivative with ammonia, the Cl is replaced by  $\text{NH}_2$ . Finally on treating the latter product with  $\text{HBr}$ , 2-methyl-5-bromomethyl-6-aminopyrimidine hydrobromide is produced.

The thiazole portion of thiamine molecule may be built up in the following manner.

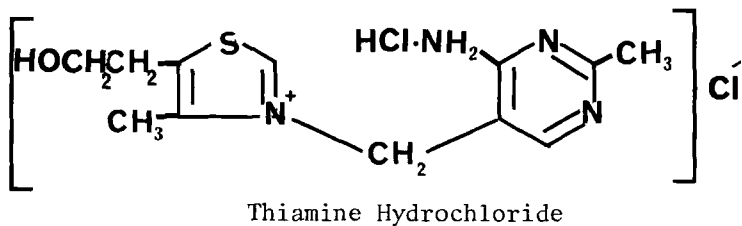
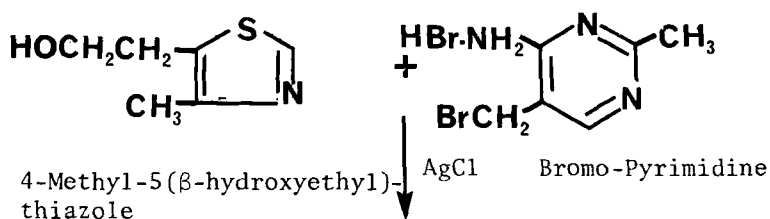
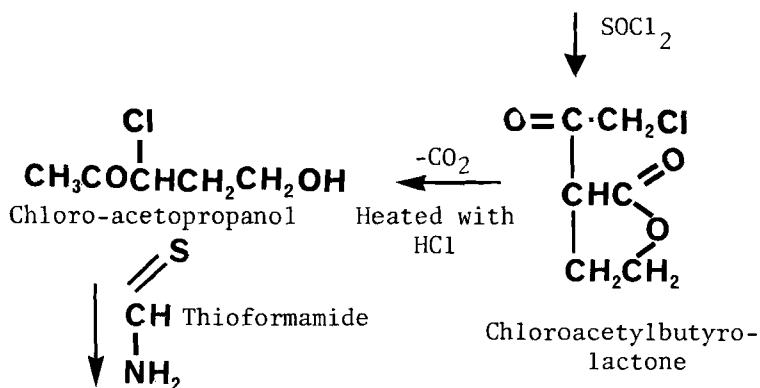
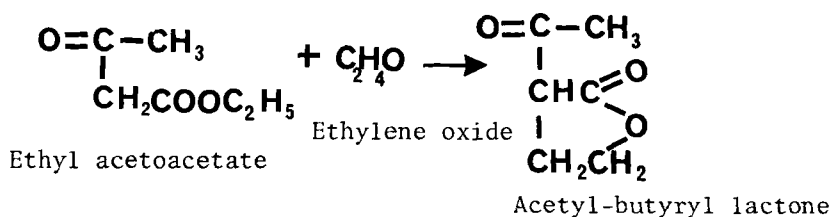
Ethyl acetoacetate [ $\text{CH}_3\text{COCH}_2\text{COOC}_2\text{H}_5$ ] is treated with ethylene oxide ( $\text{C}_2\text{H}_4\text{O}$ ) and the resulting acetylbutyryl lactone, when reacted with suluryl chloride, yields chloroacetylbutyro-lactone. This compound is decarboxylated when heated with  $\text{HCl}$ , splitting of  $\text{CO}_2$  and chloroacetopropanol. The latter when condensed with thioformamide yields the thiazol, 4-methyl-5-hydroxy ethylthiazole.

The final step of this process is the combination of the pyrimidine and the thiazol to form a thiazolium halide. Since this is a simple addition of an alkyl halide (the bromopyrimidine) to a tertiary amine (the thiazole) it is readily effected by bringing the two components together in a suitable solvent. The thiamine-bromohydrobromide so obtained is transformed into the corresponding chlorine compound thiamine with freshly precipitated silver-chloride. The silver combines with the bromine to form the less soluble

(a) Scheme II



(b) Scheme II continued



silver bromide and the chloride from the silver chloride replaces the bromine.

#### 4. Metabolism

Thiamine hydrochloride is usually given by mouth. Parenteral administration is unnecessary except in patients with impaired absorption or cardiac failure in beriberi.

Thiamine is absorbed from the gastro-intestinal tract and is widely distributed to most body tissues. It is not stored to any appreciable extent in the body and amounts in excess of the body's requirements are excreted in the urine. About 1 mg of thiamine is metabolised in the body daily and numerous metabolites are also excreted in the urine. (2)

Thiamine is fundamentally associated with carbohydrate metabolism. By combining with the pyrophosphoric acid in nucleated cells particularly in the liver, kidneys, and white blood cells, it is converted into its pyrophosphate which acts as a coenzyme in such reactions, as the decarboxylation of  $\alpha$ -ketoacids, particularly of pyruvate and  $\alpha$ -keto-glutarate. In the presence of thiamine deficiency pyruvic and lactic acids accumulate in the tissues. Thiamine pyrophosphate also acts as a co-enzyme in the direct oxidative pathway of glucose metabolism. (2)

The pyruvic acid concentration in newborn infants after prolonged labour had been reported to be elevated probably due to disturbed carbohydrate metabolism in the tissues leading to anoxia. The pyruvate concentration was reduced, though not to normal values, in infants born to mothers given 100 mg of thiamine hydrochloride intramuscularly during labour. Maternal pyruvate concentrations were not effected. (2)

Following oral administration of small doses, thiamine hydrochloride is readily absorbed, however, absorption is an active process and the total amount absorbed following oral administration of a large dose is limited to about 4-8 mg. GI absorption of thiamine is decreased in alcoholics and in patients with cirrhosis or malabsorption. The rate but not the extent of GI

absorption of thiamine is decreased when the drug is administered with meals. Thiamine is rapidly and completely absorbed following IM administration. (5)

Thiamine is widely distributed into body tissues. Body stores of thiamine have been estimated to be about 30 mg with about a 1-mg daily turnover. About 100-200 µg of thiamine is distributed daily into the milk of nursing women receiving a normal diet.

Thiamine is metabolized in the liver in animals. Several urinary metabolites of thiamine have been identified in humans. Little or no unchanged thiamine is excreted in urine following administration of physiologic doses. However, following administration of large doses, both unchanged thiamine and metabolites are excreted after stores become saturated. (5)

Thiamine is useful in correcting (at least temporarily) the metabolic disorders associated with some genetic diseases including subacute necrotizing encephalomyelopathy (SNE, Leigh's disease). Maple syrup urine disease (branched chain aminoacidopathy) and lactic acidosis associated with pyruvate carboxylase deficiency and hyperalaninemia. (5)

## 5. Vitamine Deficiency

Deficiency of thiamine results in fatigue, anorexia, gastro-intestinal disturbances, tachycardia, and irritability, and where there is reason to assume that this syndrome may be of dietary origin thiamine may be usefully employed. It may also be employed with benefit as a supplement to the diet in conditions in which thiamine deficiency may be caused by interference with its ingestion, absorption and utilization, or by increasing its destruction or excretion. Thus, its use is advisable in patients on restricted diets and those suffering from gastro-intestinal diseases. Extensive burns, diabetes, impaired kidney or liver function, hyperthyroidism, mental disease, alcoholism, and drug addiction and in those receiving antibiotics or sulphonamides over prolonged periods.

There is some evidence that alcoholic neuritis and neuritis associated with pregnancy and the neuritis of

pellagra are attributable to thiamine deficiency and will therefore benefit from thiamine hydrochloride added to the diet. Claims for its value in the treatment of other types of neuritis not associated with a vitamin B deficiency in the diet are unfounded.

Studies in obese patients suggested that thiamine deficiency readily developed in fasting obese patients. Deficiency was readily reversed by thiamine in doses as small as 500 µg daily. (2)

Thiamine is used to prevent and to treat thiamine-deficiency syndromes including beriberi. Wernicke's encephalopathy syndrome, and peripheral neuritis associated with pellagra, Wernicke's encephalopathy syndrome and high output heart failure secondary to beriberi are considered medical emergencies and IV (intravenous) or IM (intramuscular) thiamine should be administered immediately. Many clinicians recommend administration of at least 1 dose of IV or IM thiamine to all alcoholic patients with altered sensorium who are admitted to the hospital. Whenever possible, poor dietary habits should be corrected, and many clinicians recommend administration of multivitamin preparations containing thiamine in patients with vitamin deficiencies since poor dietary habits often result in concurrent deficiencies. (5)

Clinical signs of thiamine deficiency become evident after 2-3 weeks of inadequate thiamine intake. The organ systems principally affected by thiamine deficiency are the peripheral nervous system, cardiovascular system and GI tract. Administration of thiamine completely reverses the cardiovascular and GI symptoms of thiamine deficiency, however, the degree of improvement in neurological symptoms depends on the duration and severity of lesions. (5)

Feeding male rats, age 1.5-2 months a vitamin B<sub>1</sub> deficient diet over 7, 14 and 21 days decreases the cytochrome P-450 level, and aminopyrine, ethyl morphine and aniline hydroxylation rate and increases lipid peroxidation in rat liver microsomes. Supplementation of vitamin B<sub>1</sub> deficient diet with thiamine over 3 days inhibits lipid peroxidation and induces hydroxylation of xenobiotics. (6)

The thiamine deficiency effects cerebral glucose utilization. Rats maintained on a thiamine free diet for 2-7 weeks and control animals were studied by the [ $^{14}\text{C}$ ] deoxyglucose technique prior to the development of the histol lesions. This technique permits measurement of local cerebral glucose, utilization in discrete in nuclei and tracts. Levels of thiamine in brain and blood were also detected. In the 41 central nervous sytem (CNS) structures in which it was measured cerebral glucose utilization decreased with diminishing concentration of cerebral thiamine. Thus the primary metabolic consequence of thiamine deficiency is a widespread reduction in many of the structures which in humans develops histolesions with prolong thiamine defficiency than in structures less susceptible to the development of lesions. One determinant of the specific distribution of histol lesions occuring in human thiamine deficiency may be the variable rate at which the CNS structures lose their metabolic activity with continuing thiamine deficiency. (7)

## 6. Human Requirements

Owing to the fact that thiamine is not stored in the body and is rapidly lost from the tissues during short periods of deficiency, normal health cannot be maintained unless the diet regularly contains an adequate amount of the vitamin. The requirement is directly related to the carbohydrate intake and the metabolic rate. A daily intake of 200  $\mu\text{g}$  per 1000 Kcal is usually adequate to satisfy minimum requirements and tissue saturation occurs with intakes of more than 330  $\mu\text{g}$  per 1000 Kcal. The requirement is increased during periods of active growth or heavy muscular work, during pregnancy and lactation, in pathological conditions such as fevers and hyperthyroidism, and in other conditions causing increased metabolism or diuresis.

The basic recommended intake of thiamine was 400  $\mu\text{g}$  per 1000 Kcal of diet, so that men who took a diet of 3200 Kcal daily required 1-3 mg of thiamine and women who took a diet of 2300 Kcal daily required 900  $\mu\text{g}$  of thiamine. (2) The US National Research Council made the following recommendations for daily dietary allowances of thiamine:



Infants upto	2 months	200 µg
	2-6 months	400 µg
	6-12 months	500 µg
Children:	1-3 years	600 µg
	3-4 years	700 µg
	4-6 years	800 µg
	6-8 years	1 mg
	8-10 years	1.1 mg
Males:	10-12 years	1.3 mg
	12-14 years	1.4 mg
	14-18 years	1.5 mg
	18-35 years	1.4 mg
	35-55 years	1.3 mg
	over 55 years	1.2 mg
Females:	10-12 years	1.1 mg
	12-18 years	1.2 mg
	18-35 years	1 mg
	over 35 years	900 µg

In pregnancy an additional 100 µg and in lactation an additional 500 µg.

#### Cautions (Adverse effects)

Thiamine is usually non-toxic even following administration of large doses however feelings of warmth, tingling, pruritus, pain, urticaria, weakness sweating, nausea, restlessness, tightness of the throat, angioedema, respiratory distress, cyanosis, pulmonary edema, GI bleeding transient vasodilation and hypotension, vascular collapse, and death have occurred, mainly following IV administration of the drug. In animals very large parenteral doses of thiamine have produced neuromuscular and ganglionic blockade. (5)

## 7. Methods of Analysis

### 7.1 Elemental Analysis

C	42.73%	H	5.38%
Cl	21.03%	N	16.61%
O	4.74%	S	9.51%

## 7.2 Identification

A. To a volume equivalent to 20 mg of thiamine hydrochloride diluted if necessary to 10 ml of H<sub>2</sub>O for injection Or

Dissolve a quantity of powdered tablets equivalent to 20 mg of thiamine hydrochloride as completely as possible in 10 ml of H<sub>2</sub>O, and then process for both tablets and injections as: add 2 ml of dil. CH<sub>3</sub>COOH and filter add 1.6 ml of N NaOH and heat in a water bath for 30 minutes. Cool and add 5 ml NaOH solution, 10 ml of pot. ferricyanide solution and 10 ml of n-butyl alcohol, shake vigorously for 2 minutes. The alcohol layer shows an intense light blue fluorescence, especially on exposure to U.V. light. Repeat the test adding 0.9 ml of N NaOH and 0.2 g of sod. sulphite instead of 1.6 ml of N NaOH. No fluorescence is produced. (8).

B. To a volume equivalent to 50 mg of thiamine hydrochloride diluted if necessary to 5 ml of H<sub>2</sub>O in case of injections Or

Dissolve a quantity of powdered tablets equivalent to 50 mg of thiamine hydrochloride as completely as possible in 5 ml of H<sub>2</sub>O, and then process for both tablets or injections as: add 10 ml of trinitrophenol solution manipulate the ppt gently with a glass rod and allow to stand for 20 minutes. Collect the ppt and dry on porous earthenware and then at 105° for 30 minutes. MP° of the dried material, about 207° with darkening and decomposition, after sintering at about 200°. (8)

C. Dilute a volume equivalent to 50 mg of thiamine hydrochloride to 50 ml with H<sub>2</sub>O and 2 ml of HCl and heat to boiling. To the boiling solution add rapidly, drop by drop, 4 ml of freshly filtered. Silicotungstic acid solution, in case of injections Or Weigh and powder 20 tablets, or more if necessary. Add a quantity of the powder equivalent to 50 mg of thiamine hydrochloride to 60 ml of a mixture of 250 ml of H<sub>2</sub>O and 10 ml of HCl and allow to stand for one hour shaking occasionally. Filter and wash the residue with several quantities of the mix. of HCl and H<sub>2</sub>O and add to the combined filtrate and washings sufficient water to produce 100 ml. Heat 50 ml rapidly to boiling add drop by drop to the boiling solution 2 ml of freshly

filtered silicotungstic acid solution in case of tablets and then proceed for both injection or tablets as.

Boil for 4 minutes filter through a sintered glass crucible, and wash with 50 ml of boiling mixture of HCl : H<sub>2</sub>O (1:19) containing 0.2% w/v of silicotungstic acid then with two quantities each of 5 ml of acetone. Dry the residue at 105° for 1 hr cool for 10 minutes and allow to stand for 2 hrs in a desiccator over a solution of H<sub>2</sub>SO<sub>4</sub> containing 38% w/w of H<sub>2</sub>SO<sub>4</sub>. Each g of residue is equivalent to 0.1929 g of C<sub>12</sub>H<sub>17</sub>ClN<sub>4</sub>OS, HCl. (8)

(D) The identification of vit B<sub>1</sub> is based on its reaction with picrolonic acid to form an insoluble ppt in H<sub>2</sub>O. Fan-shaped crystals with two ends back in a form, specific for vit. B<sub>1</sub> are observed at a magnification of 400. Other B vitamins do not react with picrolonic acid. The sensitivity of the reaction is 0.0062 µg/µl and dilution limit is 1: 150,000. The reaction takes place in the molar ratio of the two reactants. (9)

(E) 10 ml of trinitrophenol solution is added to 5 ml of aq. thiamine solution containing 50 mg of thiamine hydrochloride. The ppt is manipulated gently with a glass rod and the mix. is allowed to stand for about 20 minutes. The ppt is dried on a porous earthenware and then at 105°C for 30 minutes its MP° is found to be 207°C with darkening and decomposition for sintering at 200°C. (10)

(F) 2 ml of dil. acetic acid and 1.6 ml of NaOH are added to 10 ml of aq. solution containing 20 mg of thiamine hydrochloride. The solution is heated for 30 minutes in a water bath and cooled to room temperature. 5 ml of NaOH solution 10 ml of 1% pot. ferricyanide solution and 10 ml of n-butyl alcohol are added and the mixture is shaken vigorously for 2 minutes. On exposure to U.V. light the alcoholic layer gives intense light-blue fluorescence on repeating the test with 0.9 ml of 0.1N NaOH and 200 mg of sod. sulphite instead of 0.1N NaOH, no fluorescence is observed. (10)

### 7.3 Titrimetric Method

(I) This method involves direct titration of thiamine salts with perchloric acid. Powder thiamine tablets are dissolved in 50 ml of glacial acetic acid. The suspension is heated on a hot plate until thiamine hydrochloride is completely dissolved leaving behind starch etc. After cooling add 10 ml of mercuric acetate solution and the solution is titrated with 0.1N perchloric acid using three drops of crystal violet indicator until the green colour appears. (11)

(II) This method is based on the reaction of vit B<sub>1</sub> with lithium picrolonate to form an insoluble ppt. of thiamine picrolonate, which is removed by filtering through a schott microfilter. Excess lithium picrolonate is titrated with methylene blue. Blank determination of lithium picrolonate is also run in the same way.

The solution containing 100 µg/ml of thiamine hydrochloride is placed in 10 ml beaker and evaporated at 60°C. 0.4 ml of saturated solution of lithium picrolonate is added, allow to stand for 2 hrs below 20°C. The ppt formed is removed by filtration through a schott microfilter. A 0.20 ml filtrate is added to mix. of chloroform and H<sub>2</sub>O (10:1) and the solution titrated against standard methylene blue. Methylene blue picrolonate formed during titration is soluble in chloroform and the end point is marked when the blue coloration in the H<sub>2</sub>O layer does not disappear after further addition of chloroform.

Amines, heavy metals and alkaline earth metals inhibit the reaction. In the presence of B<sub>2</sub>, B<sub>6</sub>, B<sub>12</sub> and nicotinic acid the solution is kept for 8 hrs for complete precipitation. 97% recoveries and method suitable for pharmaceutical preparation. (12)

### 7.4 Gravimetric Method

The method is based upon the reaction of silicotungstic acid solution with thiamine hydrochloride gravimetrically. An accurately measured assay solution corresponding to 50 mg of vitamin B<sub>1</sub> is diluted to 50 ml; H<sub>2</sub>O, 2 ml of HCl is added and solution is heated to

boiling, 4 ml of freshly filtered 10% silicotungstic acid solution is added drop by drop to the above boiling solution and heated for 4 minutes. The solution is filtered through a sintered crucible washed with 50 ml of boiling mix. of HCl and H<sub>2</sub>O (1:9, v/v), which also contains 0.2% of silicotungstic acid (w/v), followed by two 5 ml portions of acetone. The residue is dried for 1 hour at 105°C cooled for 10 minutes and allowed to stand in a desiccator over a solution of 38% H<sub>2</sub>SO<sub>4</sub> (w/w) for 2 hrs. The ppt is weighed and the amount of thiamine is calculated each gm of residue corresponds to 0.1929 g of thiamine hydrochloride. (10)

### 7.5 Volumetric Method

(I) Silicotungstic acid solution is standardized against strychnine nitrate (mol. wt. 397.2) or anhydrous thiamine hydrochloride (mol. wt. 337.3) which has previously been dried at 105°C for 2 hrs before use. A standard solution of thiamine hydrochloride containing 1 mg/ml of vitamin B<sub>1</sub> is prepared and is titrated with silicotungstic acid solution using 0.5 ml of metanil yellow as an indicator. The end point is marked when the yellow ppt changes to reddish violet through yellowish red.

The assay solution containing thiamine salts is also titrated as above. For accurate results, solution should not contain less than 0.1% vit B<sub>1</sub> and not more than 0.2% NaCl from solution of vit. B<sub>1</sub> physiological saline. With very dilute solution the end point is not sharp so 30 to 40 mg of thiamine hydrochloride should be available for titration in 15 ml of assay solution. Each milliliter of 0.05 N silicotungstic acid corresponds to 8.43 mg of thiamine hydrochloride. (13)

(II) 10 ml of the assay solution containing 100 µg to 1 mg of thiamine hydrochloride is mixed with 5 ml of 0.005N pot. ferricyanide and four drops of 20% sod. carbonate solution. After 2 minutes, 3 ml of zinc iodide solution and 3 ml of 30% acetic acid are added and the iodine liberated by excess ferricyanide is titrated with 0.002 N sod. thiosulphate.

The above procedure can also be used on a micro scale when 5 ml of assay solution containing 10-100  $\mu\text{g}$  of thiamine hydrochloride is mixed with 1 ml of 0.0025 N pot. ferricyanide. After 2 minutes, 2 ml of 1N acetic acid and 1 ml of zinc iodide solution are added and the liberated iodine is titrated with 0.001 N sod. thiosulphate. The blank determination is made and subtracted from the main titre before calculation.

(III) 10 ml of assay solution containing 100  $\mu\text{g}$  to 1 mg of thiamine hydrochloride is mixed with 5 ml of 0.005 N pot. ferricyanide and four drops of 20% sod. carbonate solution. After 2 minutes, 1 ml of 10 N HCl and two drops of indicator (5 ml D H<sub>2</sub>O contains 34.7 mg crystalline ferrous sulphate + 81.2 mg of o-phenanthroline hydrochloride) are added and the ferrocyanide in the solution is titrated with 0.002N ceric sulphate. The end point is indicated by a change in colour from orange red to green.

The method used on a microscale 5 ml of assay solution 10-100  $\mu\text{g}$  of vit B<sub>1</sub> is mixed with 1 ml of 0.0025 N pot. ferricyanide and three drops of 20% sod. carbonate solution. The solution is allowed to stand for 2 minutes 0.5 ml of 10 N HCl and 1 drops of indicator are added and the solution is titrated with 0.001N ceric sulphate. The excess ferricyanide can also be determined by titration with indigo carmine in alkaline medium. Blank determination is made and subtracted from the main titre before calculation. (14)

## 7.6 Colorimetric Method

(I) 3 ml of solution containing 300-400  $\mu\text{g}$  of vit B<sub>1</sub> is mixed with 1 ml of dil HCl (1:4) and 1 ml of sod. nitrite solution and 3 ml of saturated aq. solution of 2-(p-aminobenzene sulphonamide). Pyridine is added and the solution is shaken for 2 minutes at room temperature and made alkaline with 1 ml of KOH. The solution is allowed to stand for 5 minutes the orange colour first developed changes to pink. The solution is diluted to 25 ml in a volumetric flask, allowed to stand for 20 minutes and the absorbance is measured in 2 cm cells using filter 5-53. A calibration curve is prepared by taking different volumes of standard thiamine hydrochloride solution and by following the

above procedure. The amount of vitamin B<sub>1</sub> from the assay solution is determined by reference to the calibration curve. Ascorbic acid interferes and is oxidized with iodine before assay. (15)

(II) A measured volume containing 3-60 µg of thiamine hydrochloride is diluted to 25 ml with H<sub>2</sub>O and adjusted pH 5 with 10% acetic acid. 5 ml of diazo reagent [sod. nitrite solution + trichloroacetate of ethyl p-aminobenzoate solution (1:1)] is added and allowed to stand for 2-3 minutes before it is made alkaline with 1N NaOH, again allowed to stand for 2 minutes so that maximum intensity of colour is developed. 5 ml of isoamyl alcohol is added, the mixture is shaken thoroughly and two layers are allowed to separate. The alcoholic layer is removed, dried over anhydrous sod. sulphate and the absorbance is measured with colorimeter. A calibration curve is prepared by taking different volumes of standard thiamine solution and by following the above procedure. The amount of vit B<sub>1</sub> from the assay solution is determined by reference to the calibration curve. (16)

#### 7.7 Spectrophotometric Method

##### Visible Absorption Spectroscopy

(I) In this method 10 ml of solution in acetate buffer containing 2.5 mg of thiamine hydrochloride is mixed with 5 ml of amm. reineckate allowed to stand for 30 minutes and filtered through sintered crucible, ppt. washed with 3 ml portions of wash solution applying gentle suction, dried and dissolved in 10 ml of acetone, the extinction is determined at 525 nm against H<sub>2</sub>O as blank. A calibration curve is prepared by taking 1, 2, 3, 4 and 5 ml of thiamine hydrochloride reference solution diluted to 10 ml with pH 4.5 acetate buffer and by following the above procedure. The amount of vit B<sub>1</sub> from the assay solution is determined by reference to the calibration curve. (17)

(II) To 1 ml of solution containing 5-60 µg of vit B<sub>1</sub> 0.2 ml of stabilized diazonium norsulphazol salt solution and 0.2 ml NaOH are added followed by 2 ml of basic solution. The solution is stirred several times with an interval of few minutes. The volume is made up

to 5 ml with H<sub>2</sub>O and the absorbance of pink colour is measured at 485 nm with 5-10 minutes. A calibration curve is prepared by taking standard thiamine solution containing 5-60 µg/ml of vit B<sub>1</sub> and by following the above procedure. The amount of thiamine hydrochloride from the assay solution is found by reference to the standard curve. (18)

Recently the above procedure is used for the determination of 0.6 and 5% thiamine solution in ampoules. 1 ml of freshly prepared diazotized norsulphazol solution is added to 0.2-0.5 ml of 0.6% thiamine hydrochloride solution followed by 2 ml of 1N NaOH and solution is diluted to 50 ml with D. H<sub>2</sub>O. After 10 minutes absorbance is measured using a blue-green filter against a solution containing 1 ml of diazotized norsulphazol and 2 ml of 1N NaOH in 50 ml. (19)

(III) 10 ml of assay solution is mixed with 10 ml of  $1 \times 10^{-4}$  dye solution in a separating funnel by vigorous shaking for about 2 minutes. The chloroform is collected while the aqueous layer is extracted twice more with 10 ml of chloroform and the three chloroform extracts are combined. The solution is evaporated to about 7 ml at 60°C, then cooled to room temperature and diluted to 10 ml with chloroform, centrifuged at 2500 r.p.m. for 5 minutes then the absorbance of clear solution is measured at 420 nm. The blank is prepared by substituting 10 ml of plain buffer solution for 10 ml of thiamine solution. The amount of thiamine is determined from the calibration curve which is prepared by using different amounts of pure thiamine and by following the above procedure. (20)

#### UV Absorption Spectroscopy

(I) In the UV region thiamine hydrochloride exhibits marked absorption which is pH dependent and is used for the determination of thiamine injections and pure solutions.

The assay sample is adjusted to either pH 2 with 1N HCl or to pH 7 with phosphate buffer and diluted with D. H<sub>2</sub>O to obtain a solution containing 10-20 µg/ml of vit B<sub>1</sub>. The extinction of the solution is measured



spectrophotometrically in 1 cm cells against H<sub>2</sub>O as blank at 246 or 232 nm depending upon the pH of the solution.

Amount of thiamine hydrochloride per 100 ml of solution in mg at pH 2 =  $E_{246} \text{ nm} / 0.425$ . (21)

(II) In this method the vit B<sub>1</sub> from multivitamin preparations is separated on a column of 'Zeokorb' 226 and the column adsorbing vit B<sub>1</sub> is then eluted with dil. HCl and the elute is assayed at 246-247 mμ. The tablets are dissolved in D. H<sub>2</sub>O and the solution diluted to certain volume, centrifuged and an aliquot of the clear solution (also from the liquid preparation can be used) containing 0.5-5.5 mg of vit B<sub>1</sub> is sufficiently diluted with D. H<sub>2</sub>O to have an easy flow and is percolated through the column followed by 200 ml of H<sub>2</sub>O. The vitamin adsorbed on the column is then eluted with 200 ml of 0.1N HCl and thiamine is determined spectrophotometrically at 247 mμ using blank acid elute in the blank cell. The resin column can be used again several times. (22)

### 7.8 Polarographic Method

Several methods are known for the determination of thiamine by polarography. These methods are based on the measurements of a reduction wave in alkaline solutions and of a catalytic wave in cobalt salt solution or unbuffered solution. The anodic wave at pH 9 is particularly suitable for quantitative assay, which is due to the formation of a mercury salt from the thiol form of thiamine. The wave height and equilibrium concentration of the thiol form are altered with pH, the half wave potential is - 0.42 V. The wave height is maximum at pH 12, and is proportional to the concentration between 50 and 200 μg/ml of vit B<sub>1</sub>. (23)

Deryatnin has also developed a polarographic method for determination of vit. B<sub>1</sub> is carried out in a 3% KOH solution. The half wave potential is - 0.45 V. The other vitamins present in multivitamin preparations do not interfere and the method has been recommended for industrial controls due to its quickness and precision. (24)

A method for estimating thiamine and its monophosphate and pyrophosphate esters in the same sample is proposed. The anodic waves of thiamine and its mono and pyrophosphates appear at  $E_{\frac{1}{2}}$  values of - 0.36 to - 0.4 V vs. SCE at pH > 9.0, 9.3 and 9.6 respectively. Careful adjustment of pH in sep. aliquots to pH 9.0-9.3, 9.3-9.6, and > 9.6 allows measurements of waves corresponding to thiamine and its monophosphate, and pyrophosphate respectively. Samples containing thiamine disulfide can also be analysed. (25)

### 7.9 Fluorimetric determination

1. The thiochrom method for determination of thiamine in pharmaceutical preparations was adapted to a continuous flow system based on the flow injection principle. The sample volume required for an analysis is about 150  $\mu$ l and for routine purposes a concentrations ranges of  $3 \times 10^{-4}$  -  $6 \times 10^{-4}$  mg/ml is used. Results obtained with the system agree well with the results obtained manually. The consumption of organic phase is 2-3 ml/sample and the sampling rate is 30/h. A sampling rate of 70/h is easily attained if necessary, the relative standard deviation is about 1%. (26).

2. Thiamine is detected in brewers yeast by fluorometry of yeast extractions. Yeast are extracted with an HCl solution and the extract is defecated and diluted to a suitable concentration. Thiamine is oxidised to thiachrome with  $K_3Fe(CN)_6$ . Thiachrome is then extracted with isobutanol and determined by its fluorescence at 435 nm with excitation at 365 nm. (27)

3. An improved method for electrophoretic separation and fluorometric (or radiometric) determination of thiamine and thiamine mono-, and triphosphates in animal tissues (liver, small intestine, kidney, heart, brain) is described. The amount of total thiamine handled was 1.5-3.5  $\mu$ g. The procedure includes, acid extraction of the compounds from the tissue, deproteinization with TCA, purification of the extract, on partially, deactivated charcoal elution with 10% Propanol in 0.1N formic acid, lyophilization,, electrophoresis on gelatinized cellulose acetate, strips elution of the thiamine bands with 50% ethyl

alcohol and finally oxidation to thiochromes and reading of eluate fluorescence. The mean internal recovery was 83.3% and the external one 83.5%. Dephosphorylation of the thiamine phosphates was rather slight and each thiamine compound was comparably recovered (82.0-87.7%) apparently thiamine triphosphate being the worst and thiamine the best recovered compound. (28)

#### 7.10 A.A. Spectroscopy

The determination of B<sub>1</sub> in pharmaceutical preparations using a lead ion selective electrode and atomic absorption spectroscopy based on its reaction with 0.02 M alk. plumbile, under absorption spectroscopy (217 nm) or by titration with EDTA (pH 4.5) with Pb ion. Selective electrode and Gran's plot KOH is the preferred alkali in the desulfurization reaction. The results obtained by this method compared favourably with those obtained by USP fluorimetric method. Other vitamins and excipients did not interfere with the electrode or absorption spectroscopy procedures. Recoveries averaged 99.1% and 99% for the spectroscopy and electrode methods respectively and standard deviations were 0.8% and 0.7% respectively. (29)

#### 7.11 Chromatographic Methods

##### Paper Chromatography

Thiamine hydrochloride is separated from Vit. B<sub>6</sub> and other ingredients of multivitamin preparations on a chromatographic paper (Schleicher-Schiell 2043b MgI) impregnated with phospho-citrate buffer of pH 3.5. Butyl alcohol saturated with water is used as a developing solvent. A mixture containing 1.5 ml of 1% potassium ferricyanide, 3 ml of 15% sodium hydroxide, and 20 ml of water is used as a spray reagent. The spots are eluted with water and extinction of eluate is measured at 270 nm. (30).

### Gas Chromatography

Gas chromatographic methods have been used for the determination of thiamine hydrochloride and are summarised in the Table (4).

### Thin-layer Chromatography (TLC)

A summary of some of the TLC systems investigated for the analysis of thiamine hydrochloride are given in the Table (5).

### High Performance Liquid Chromatography

High pressure liquid chromatography HPLC method has wide application for the estimation of thiamine hydrochloride. A summary of variable parameters in a few cases is given in Table (6).

## 8. Acknowledgment

The authors would like to thank Mr. Tanvir A. Butt, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia for typing the manuscript.

## 9. References

1. The "Merck Index", 10th Edition, p. 9134, 1983.
2. The Extra Pharmacopoeia "Martindale" 26th Edition, the Pharmaceutical Press, London.
  - 2a. Unpublihsed data, Mohammad Saleem Mian, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia.
  - 2b. "CRC Atlas of Spectral Data and Physical Constants of Organic Compounds", edited by J.G. Grasselli, CRC Press, Cleveland, Ohio, Vol. III, 1975.
3. "The Vitamins" Vol. III, W.H. Sebrell, Jr. and Robert S. Harris. Academic Press, Inc., New York, 1954.
4. "Remington's Pharmaceutical Sciences", 13th Edition, Mack Publishing Company, Easton, Pennsylvania, p. 1102. 1965.

Table (4). Summary of conditions used for G.C. of vitamin B<sub>1</sub>

Column Support	Mesh	Temp.	Flow rate	Sample	Ref.
0.325% (w/w) EGA AW H1 Chromosorb	80-100	75°C	Carrier gas Helium 60 ml/min H <sub>2</sub> 50 ml/min	Plasma	31
5% OV-17 Chromosorb WAW DMCS	80-100	150°C	18 ml/min		32

Table (5) Summary of conditions used for the TLC of vitamin B<sub>1</sub>

Plate	Developing Solvent	Detection	Extd. Solvent	Rf	Ref.
Silica gel G 0.25 mm thick	Me <sub>2</sub> Co-MeOH-C <sub>6</sub> H <sub>6</sub> (1:2:8)	Densitometer	-	-	33
Silica gel G	CHCl <sub>3</sub> -EtOH-H <sub>2</sub> O (50:25:1)	Spectrophotometer UV 246 nm	2N HCl	-	34
448 Silica gel GF254	-	Densitometer	-	0.057±0.01	35
Silica gel GF254	Diethanolamine: Methanol: formic acid: basic Na. phosphate (1:15:1.5:5)			0.56	36

Continued (Table 5)

Plate	Developing Solvent	Detection	Extd. Solvent	Rf	Ref.
Polyamide layers	Acetone: diethyl- ether: glacial acetic acid (20:20:1)	Iodoplatinate or Iodine vapor	-	-	37
	Chloroform: ethyl- acetate: glacial acetic acid (20:10:1)		-	-	
Ferligfolien	D. H <sub>2</sub> O	Expose dried Chromatogram to Cl. Then spray o toli- dine KI reagent UV 254	-	-	38

Table (6). Summary of HPLC conditions for the determinations of vitamin B<sub>1</sub>

Column	Mobile phase	Flow rate	Retention time	Sample	Detection	Ref.
Zipax Scx.	0.05 M Na <sub>2</sub> HPO <sub>4</sub> (pH 9) and 0.8 M NaClO <sub>4</sub>	-	-	Capsule extract	UV photo-meter	39
-	0.2 M NaH <sub>2</sub> PO <sub>4</sub>	0.5 ml/min	-	Deproti-nized blood supernatant adjusted to pH 4.5 with NaOAc.	Spectro-fluorometer	40
μ-Bondapack C <sub>18</sub>	CH <sub>3</sub> CN: H <sub>2</sub> O (70:30)	-	-	Blood plasma	Fluori-metry	41
Shimadzu ISA-07/52504 LC column (25 mm X 0.4 mm) ID	0.7 M sod. ace-tate	0.5 ml/min	-	Nervous tissues	Spectro-fluorometer	42



## Continued (Table 6)

Column	Mobile phase	Flow rate	Retention time	Sample	Detection	Ref.
Radial-PAK B cartridge (silica; 10 $\mu$ m)	Phosphate buffer solution: methanol (7:3)	3 ml/min	-	Acidified urine	Flourescence	43
Column (10 cm X 18 mm) ID Radial-PAK C <sub>8</sub> (10 $\mu$ m) and guard column of Bondapak C <sub>18</sub> /Porasil	37% methanol 0.1M phosphate buffer (pH 7.0)	15 or 3 ml/min	-	Food	Flouri-metric 530 nm	44
Nucleosil C <sub>18</sub> (5 $\mu$ m)	Methanol: H <sub>2</sub> O (19:1)	-	-	Bloodor urine	Flouri-metric	45

Continued (Table 6)

Column	Mobile phase	Flow rate	Retention time	Sample	Detection	Ref.
8 $\mu$ Bondapak C <sub>18</sub>	3 to 8 mM Na hexanesulphonate in aq. 25% methanol containing 1% of acetic acid	1.0 ml/min	-	Powdered tablets or injections	254 nm	46
LiChrosorb RP-8	Methanol-acetonitrile-isobutyl alcohol (8:1:1)	-	-	Food	Spectro-fluorimetric 425 nm.	47
(30 cm X 3.9 mm) of $\mu$ Bondapak phenyl (10 $\mu$ m)	0-80% of methanol in H <sub>2</sub> O	2.0 ml/min	-	Multi-vitamin tablets	280 nm	48
(30 cm X 4 mm) of $\mu$ Bondapak C <sub>18</sub>	0.2M acetate buffer with 5 mM heptane sulphonic acid	1.0 ml/min	-	Food	UV 250 nm	49

Continued (Table 6)

Column	Mobile phase	Flow rate	Retention time	Sample	Detection	Ref.
Stainless steel column (50 cm X 2.1) mm packed with Spherisorb silica (20 µm)	CH <sub>3</sub> Cl: methanol (9:1)	1.0 ml/min or 0.8 ml/min	-	Meat	Flourimetry 367	50
Stainless steel column (30 cm X 4 mm) packed with µ Bondapak C18 (10 µm)	Methanol-aq. 5 mM hexanesulphonate containing 1% of acetic acid (1:3)	0.5 ml/min	-	Multi-vitamin	UV 270 nm	51

Continued (Table 6)

Column	Mobile phase	Flow rate	Retention time	Sample	Detection	Ref.
Two columns (50 cm X 21 mm) con- nected in series and packed with HS Pellionex SCX.	0.1M phosphate	10-20 ml/hr	-	Multi- vitamin tablets	UV 254 nm or 280 nm	52

5. "Drug Information 88". American Society of Hospital Pharmacists.
6. Sushko, L.I.; Lukienko, P.I., Farmakol Toksikol, (Moscow) 44(1) 102-4 (Russ) 1981.
7. Hakim, A.M.; Pappius, H.M. Ann. Neurol, 9(4), 334-9 (Eng.) 1981.
8. "The British Pharmacopoeia" Her Majestys' Stationery Office, London, p. 471, 1973.
9. Assay of Vitamins in Pharmaceutical Preparations Manzur-Ul-Haque Hashmi. John-Wiley & Sons, Chichester, New York.
10. British Pharmacopoeia, p. 1010. 1968.
11. C.W. Pirfer and E.G. Wallish. J. Am. Pharm. Assoc., 40, 609 (1951).
12. P. Vasseileva-Alexendrova and A. Alexandrov. Mikrochim. Acta, 277 (1968).
13. E. Graf and E. Fiedler, Arzneimittel Forsch, 3, 489 (1953).
14. H. Wachsmuth. Bull. Soc, Chim, Belges, 56, 261 (1947).
15. C.S. Runti, Intern. Z. Vitaminforsch, 19, 282 (1948).
16. E.R. Kirch and O. Bergeim. J. Biol. Chem., 143, 575 (1942).
17. F.J. Bandelin and J.V. Tuschhoff. Anal. Chem., 25, 1198 (1968).
18. A.M. Aliev. Aptechn. Delo, 12(2), 50, 1963.
19. A.M. Aliev and F.M. Akmedova. Azerb. Med. Zh. 48, 39 (1971).
20. V.D. Gupta and D.E. Cadwallader. J. Pharm. Sci. 57, 112 (1968).

21. J.J. Doherty, N. Cane and F. Wokes. J. Pharm. Pharmacol, 7, 1053 (1955).
22. M. Chakraborty and S.K. Dutta. J. Inst. Chem., India, 39, 288 (1967).
23. R. Pleticha, Pharmazie, 12, 219 (1957).
24. V.A. Devyatnin and L.A. Kuznetsova, Med. Prom. USSR, 18(3), 58 (1964).
25. Vergara, T., Marin, D.; Vera, J. Anal. Chim. Acta, 120, 347-51 (Eng), 1980.
26. Karlberg, B.O., Thelander, Sidsel, Ann. Chim, Acta, 114, 129-36 (Eng), 1980.
27. Batista, Maria, Teresa, P.M.; Baeta, Maria-de Lurdes, M.A. (Port) Bol. Fac. Farm., Coimbra 3(1), 41-3 (Port), 1979.
28. Patrini, C., Rindi, G. Int. J. Vitam. Nutr. Res. 50(1), 10-18 (Eng), 1980.
29. Hassan, Saad, S.M.; Zaki, Mohamed T., Eldesouki, Mohamed, H. J. Assoc. Off. Anal. Chem. 62(2), 315-19 (Eng), 1979.
30. V. Kohen and N. Russeva, Farmatsiya (Sofia), 19(1), 4, (1968).
31. Hilker, D.M.; Mee, John, M.L., J. Chromatogr., 76(1), 239-41 (Eng), 1973.
32. Richard, E. Echols, Toubert Harris, and Robert, H., Miller, J. of Chromatography, 1983, 470-475.
33. Kouimtzis, T.A., Papadoyannis, I.N. Mikrochim. Acta, 1(1-2), 145-9 Eng. 1979.
34. Ismaiel, Saad, A., Yassa, Dawoud, A. Analyst (London) 98(1162), 5-8 (Eng), 1973.
35. Cadorniga, R. Dominguez, Gil. A., Conzalez, M.C. Farmco, Ed. Prat. 27(10), 572-84, (Spain), 1972.

36. C. Levorato and L. Cina. *J. Chromatog.*, 32, 77 (1968).
37. H.P. Chuang, H.C. Chiang and K.T. Wang. *J. Chromatogr.*, 41, 487 (1969).
38. Thielmann, Horst *Pharmazie*, 35(2), 125 (in German), 1980.
39. R.C. Williams, D.R. Baker, and J.A. Schmit, *J. of Chromatographic Science*, Vol. 11, 618-624, Dec. 1973.
40. Kimura, Mieko;; Fujita, Tomio, Itokawa, Yoshinori, *Bitamin* 55(4), 185-9, Japan, 1981.
41. Hisaoka, Masafumi, Terao, Toshio, Kojima, Toshimaso, Morioka, Tadashi, Sankyo, Kenkyusho, Nempo, 32, 98-103, Japan, 1980.
42. Miekokimura, Tomiofujita, Shigeki, Nishida and Yoshinori, Itonawa, *J. of Chromatography* 188, 417-419, Elseveir Scientific Publishing Co. Amsterdam. Printed in Netherland, 1980.
43. Mansourian, R. Barclay, D. and Dirren, H. *Int. J. Vitam. Nutr. Res.* 52(2), 228, 1982.
44. Fellman, J.K.; Artz, W.E.; Tassinari, P.D.; Cole, C.L. and Augustin, J. *J. Food Sci.* 47(6), 2048-2050, 2067, 1982.
45. Yasuda, Kazuto; Ikeda, Ritsuko; and Kawada, Akiko *Rinsho Byori*, 29(6) 564-568 (in Japanese) 1981.
46. Walker, M.C.; Carpenter, B.E. and Cooper, E.L., *J. Pharm. Sci.*, 70(1), 99-101, 1981.
47. Bogнар, Antal, *Dtsch. Lebensm. Rundsch.* 77(12) 431-436 in German, 1981.
48. Kwok, Roderic, P.; Rose, W.P., Tabor, Rick; and Paltison, Thomas, S. *J. Pharm. Sci.*, 70(9), 1014-1017, 1981.
49. Skurray, Geoffre, R. *Food Chem.* 7(2), 77-80, 1981.

50. Ang, Catharina, Y.W., and Moseley, Frederick, A.J. Agric. Food Chem. 28(3), 483-486, 1980.
51. Kirchmeier, R.L.; and Upton, R.P., J. Pharm. Sci., 67(10), 1444-1446, 1978.
52. Callmer, K.; and Davies, L. Chromatographia, 7(11), 644-650, 1974.



ANALYTICAL PROFILE  
OF  
THIORIDAZINE  
THIORIDAZINE HYDROCHLORIDE

Ezzat M. Abdel-Moety and Khalid A. Al-Rashood

Department of Pharmaceutical Chemistry,  
College of Pharmacy, King Saud University,  
P.O. Box 2457, Riyadh-11451,  
Saudi Arabia.

## CONTENTS

### 1. INTRODUCTORY

### 2. DESCRIPTION

- 2.1 Nomenclature
- 2.2 Formulae and Molecular Weight
- 2.3 Appearance, Color, Odor and Taste
- 2.4 The Three-Dimensional Structure

### 3. PHYSICAL CHARACTERISTICS

- 3.1 Elemental Composition
- 3.2 Acidity (pH)
- 3.3 Ionization Constant (pKa)
- 3.4 Melting Range and Boiling Point
- 3.5 Thermal Behavior
- 3.6 Solubility
- 3.7 Crystallographic Data
- 3.8 Spectroscopic Data
  - 3.81 Ultraviolet (UV) Absorption
  - 3.82 Infrared (IR) Spectroscopy
  - 3.83 Mass Spectroscopy (MS)
  - 3.84 Nuclear Magnetic Resonance (NMR)
    - 3.841  $^1\text{H}$ -NMR Spectrum
    - 3.842  $^{13}\text{C}$ -NMR Spectrum

### 4. SYNTHESIS

- 4.1 Manufacturing
- 4.2 Partial Synthesis
- 4.3 Cyclization

### 5. PHARMACOKINETICS

- 5.1 Absorption
- 5.2 Distribution
- 5.3 Biotransformation
- 5.4 Drug Concentration Levels
- 5.5 Elimination

### 6. THERAPEUTIC CATEGORATION

- 6.1 Pharmacology

- 6.2 Uses
- 6.3 Drug-Drug Interactions
- 6.4 Toxicology
- 6.5 Cautions
- 6.6 Administration and Dosage
- 6.7 Pharmaceutical Preparations
- 6.8 Stability
- 6.9 Laboratory Test Interferences

## 7. ANALYTICAL METHODS

- 7.1 Qualitative
- 7.2 Quantitative
  - 7.21 Determination in Bulk Materials
  - 7.22 Determination in Pharmaceutical Formulations
  - 7.23 Determination in Tissues and Biological Fluids

## ACKNOWLEDGEMENT

## REFERENCES

## 1. INTRODUCTORY

Much attention has been given to a group of psychotropic drugs because of the increasing abuse of these drug substances for suicides and use as narcotics. The antipsychotic phenothiazine group has a variety of derivatives to which our drug thioridazine belongs. Thioridazine is an alkylpiperidine derivative of the prototype phenothiazine. The drug was synthesized firstly in the year 1958 and has been patented (U.S. Patent : 3.239.514) to Sandoz Ltd., Basel-Switzerland. Since then huge numbers of publications have appeared concerning the clinical (therapeutic and pharmacokinetic) and chemical characteristics of the drug. This work is a trial to summarize and integrate the net findings of such investigations collectively in a useful profiling way; any gap is not intended of course.

## 2. DESCRIPTION

### 2.1 Nomenclature

#### 2.11 Systemic Name

Thioridazine : 10-[2-(1-Methyl-2-piperidyl)ethyl]-2-(methylthio)phenothiazine (1).

Thioridazine Hydrochloride : 10-[2-(1-Methyl-2-piperidyl)ethyl]-2-(methylthio)phenothiazine monohydrochloride (1).

#### 2.12 Other Chemical Names

Thioridazine is 2-methylmercapto-10-[2-(N-methyl-2-piperidyl)ethyl]phenothiazine; 3-methylmercapto-N-[2'-(N'-methyl-2-piperidyl)ethyl]phenothiazine; 1-methyl-2-[2-(2-methylthiophenothiazine-10-yl)ethyl]piperidine (2); or 10-[2-(1-Methylpiperid-2-yl)ethyl]-2-methylthiophenothiazine (3).

#### 2.13 Pharmacopoeias

Thiordazine in Nord.P. and U.S.P.

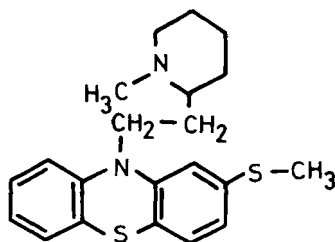
Thioridazine.HCl in B.P., Cz.P., Jug. P., Nord.P., and U.S.P.

## 2.14 Chemical Abstracts Registry (CAS) Number

Thioridazine : [50-52-2].

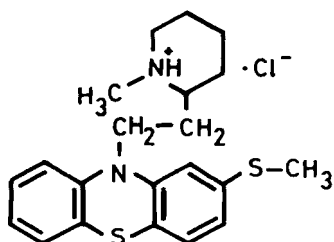
Thioridazine.HCl : [130-61-0].

## 2.2 Formulae and Molecular Weight



(Thioridazine)

$C_{21}H_{26}N_2S_2$ ; MW: 370.56



(Thioridazine.HCl)

$C_{21}H_{26}N_2S_2 \cdot HCl$ ; MW: 407.02

Thioridazine is commercially available as the base and as the hydrochloride salt. Each 110 mg of thioridazine hydrochloride is approximately equivalent to 100 mg of thioridazine.

## 2.3 Appearance, Color, Odor and Taste

Thioridazine and its HCl-salt are white to slightly yellow crystalline powder with a faint odor and a very bitter taste (4). The powder darkens on exposure to light (5).

## 2.4 The Three-Dimensional Structure

Each two molecules in the unit cell of the drug have slightly different conformation (6). The angles between the two phenyl planes are  $135^\circ$  and  $145^\circ$  respectively (7).

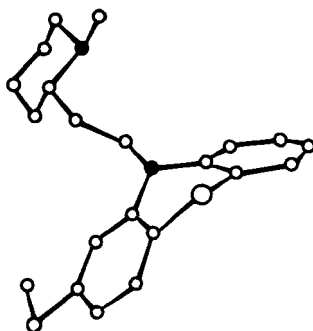


Fig.1 : The three-dimensional structure of thioridazine.

### 3. PHYSICAL CHARACTERISTICS

#### 3.1 Elemental composition

<u>Elemental</u>	<u>Thioridazine</u> (%)	<u>Thioridazine.HCl</u> (%)
C	68.06	61.96
H	7.07	6.44
Cl	—	8.96
N	7.56	6.88
S	17.31	15.76

#### 3.2 Acidity (pH)

The determined pH-value of 1% aqueous solution of thioridazine HCl was between 4.2 and 5.2 (2,5,8).

#### 3.3 Ionization Constant (pKa)

The pKa-value of the base thioridazine was determined to be 9.5 (5,9,10).

#### 3.4 Melting Range and Boiling Point

	<u>Melting Range, °C</u>	<u>Boiling Point, °C</u>
<u>Thioridazine</u>	72-74 (3-5)	230 (0.02 mmHg) (3,4)
<u>Thioridazine</u>	157-163 (1,5)	—
<u>hydrochloride</u>	159-163 (2,3)	
	158-160(4).	

### 3.5 Thermal Behavior

The differential scanning calorimetry (DSC) thermal curve for thioridazine hydrochloride is given in Figure 2. The scanning has been run at a rate of  $10^{\circ}\text{C}.\text{min}^{-1}$  from 50 to  $200^{\circ}\text{C}$ . The hydrochloride salt of thioridazine melts at  $166.8^{\circ}\text{C}$ , the  $\Delta H$ -value is  $44.2 \text{ J}.\text{mole}^{-1}$  for 95.84 mole% purity. A DuPont TA-9900 Thermal Analyzer attached to a DuPont Data Unit were used for the DSC-scanning.

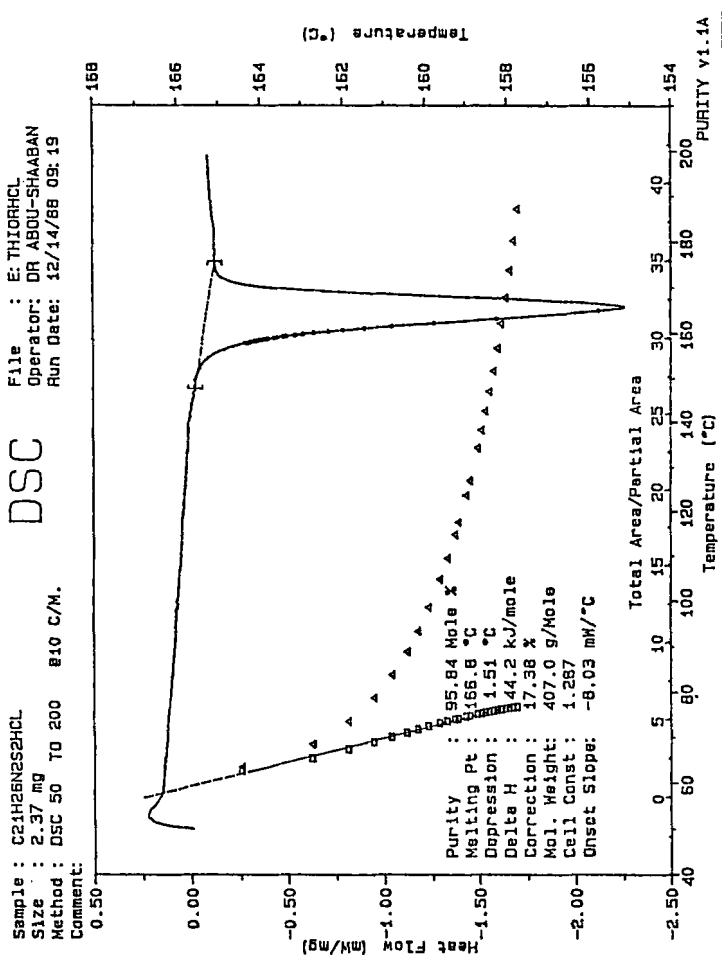


Fig. 2 : The differential scanning calorimetric (DSC) thermal curve of thioridazine hydrochloride.

### 3.6 Solubility

The base thioridazine is water insoluble but it is soluble in alcohol, (1 in 6), in chloroform (1 in 0.81), in ether (1 in 3), and freely soluble in dehydrated alcohol (5). Thioridazine hydrochloride dissolves in water (1 in 9), freely soluble in methyl alcohol, in ethanol (1 in 10), in chloroform (1 in 5), but it is insoluble in ether (3).

### 3.7 Crystallographic Data

#### 3.71 Crystallization

The free base thioridazine as well as monohydrochloride salt crystallize from acetone (4).

#### 3.72 Crystal Forms

Figure 3 demonstrates the different crystalline forms of thioridazine hydrochloride; the crystal tracing was undertaken in glycerine mount by using a Leitz Camera Lucida ( $X = 40$ ) attached to a Leitz projector. A stage scale micrometer was utilized under the same magnification.

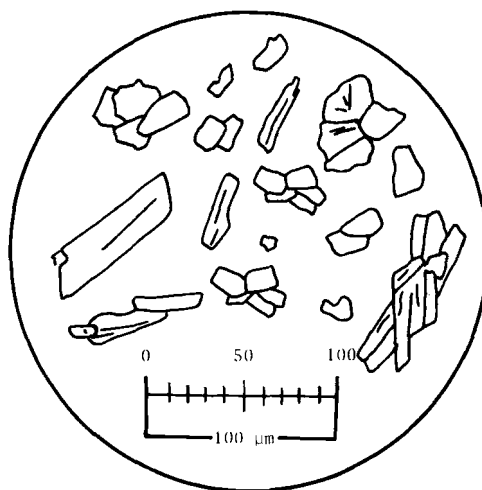


Fig. 3: Microscopic examination of different crystal forms of thioridazine hydrochloride.



### 3.73 X-Ray Diffraction Pattern

This pattern was obtained on a Philips PW-1710 Diffractometer with single crystal monochromator and copper  $K_{\alpha}$  radiation. The x-ray powder diffraction patterns were recorded on a Philips PM-8210 printing recorder. The tables of  $2\theta$ , d-spacing ( $\text{\AA}$ ), and counts were automatically obtained on a Philips Digital printer.

Table 1: The X-ray diffractational principal lines of thioridazine hydrochloride.

$2\theta$	d( $\text{\AA}$ )	[I/I <sub>0</sub> x 100]	$2\theta$	d( $\text{\AA}$ )	[I/I <sub>0</sub> x 100]
5.104	17.3150	100	32.611	2.745	10.8
7.367	11.999	24.4	33.063	2.709	6.8
10.073	8.781	15.2	33.474	2.676	7.4
13.253	6.680	46.4	34.582	2.593	10.8
15.638	5.666	46.9	36.541	2.459	10.8
15.914	5.568	30.7	37.035	2.427	8.1
16.375	5.413	19.4	38.369	2.346	4.1
17.267	5.135	36.2	39.127	2.302	9.4
17.891	4.957	19.9	39.465	2.283	7.1
18.488	4.799	7.1	40.404	2.232	4.7
19.550	4.540	19.1	41.090	2.196	7.1
19.964	4.447	40.8	42.119	2.145	7.4
21.178	4.195	35.5	42.767	2.114	11.0
21.613	4.111	8.0	44.247	2.047	6.6
23.555	3.776	12.8	44.578	2.032	8.6
24.260	3.668	10.4	45.535	1.992	4.3
24.876	3.579	45.2	46.144	1.967	5.3
25.330	3.516	35.3	46.465	1.954	4.6
26.044	3.421	10.6	48.306	1.884	4.8
26.904	3.313	17.1	49.298	1.848	4.8
27.770	3.212	10.8	51.336	1.779	5.9
28.491	3.132	8.6	53.138	1.723	4.1
29.302	3.047	17.6	53.687	1.707	4.5
30.495	2.931	6.8	54.121	1.694	4.4
30.866	2.896	6.5	55.438	6.657	4.1
31.514	2.838	7.6	56.916	1.617	3.6
32.292	2.772	7.8			

Figure 4 shows the characteristic principal lines of the X-ray powder diffraction of thioridazine hydrochloride.

### 3.8 Spectroscopic Data

#### 3.81 Ultraviolet (UV) Absorption

The UV-measurements were undertaken for thioridazine HCl solutions in water, 95% ethanol, 0.1N NaOH, and 0.1N HCl against the corresponding solvent using matched 1-cm quartz cells. A UV-visible Varian DMS-90 double beam Spectrophotometer, attached to a Hewlett-Packard 7015 B X-Y recorder were used. The  $A(1\%, 1\text{cm})$ -values and the corresponding molar absorptivities of the drug are collectively summarized in Table 2. Figure 5 represents the obtained UV-spectra.

Table 2 : The UV-spectral characteristics of thioridazine base and its hydrochloride salt.

Solvent	Thioridazine (3)			Thioridazine.HCl		
	(nm)	$A(1\%, 1\text{cm})$	$\epsilon$	(nm)	$A(1\%, 1\text{cm})$	$\epsilon$
Water	--	--	--	262	1028	41842
				310	79	3215
95% ethanol	263	1030	38172	264	1022	41598
	314	124	4595	310	80	3256
0.1N Acid	230	565	20939	264	1041	42371
	263	1240	45954	305	135	5495
0.1N Alkali	313	141	5226	263	452	18392

#### 3.82 Infrared (IR) Spectroscopy

The IR-spectrum of thioridazine hydrochloride as KBr-disc was made on a Perkin Elmer 580B Infrared Spectrometer. Figure 6 shows the obtained IR-spectrum, while Table 3 illustrates the correction of the structural assignments with the recorded band frequencies.

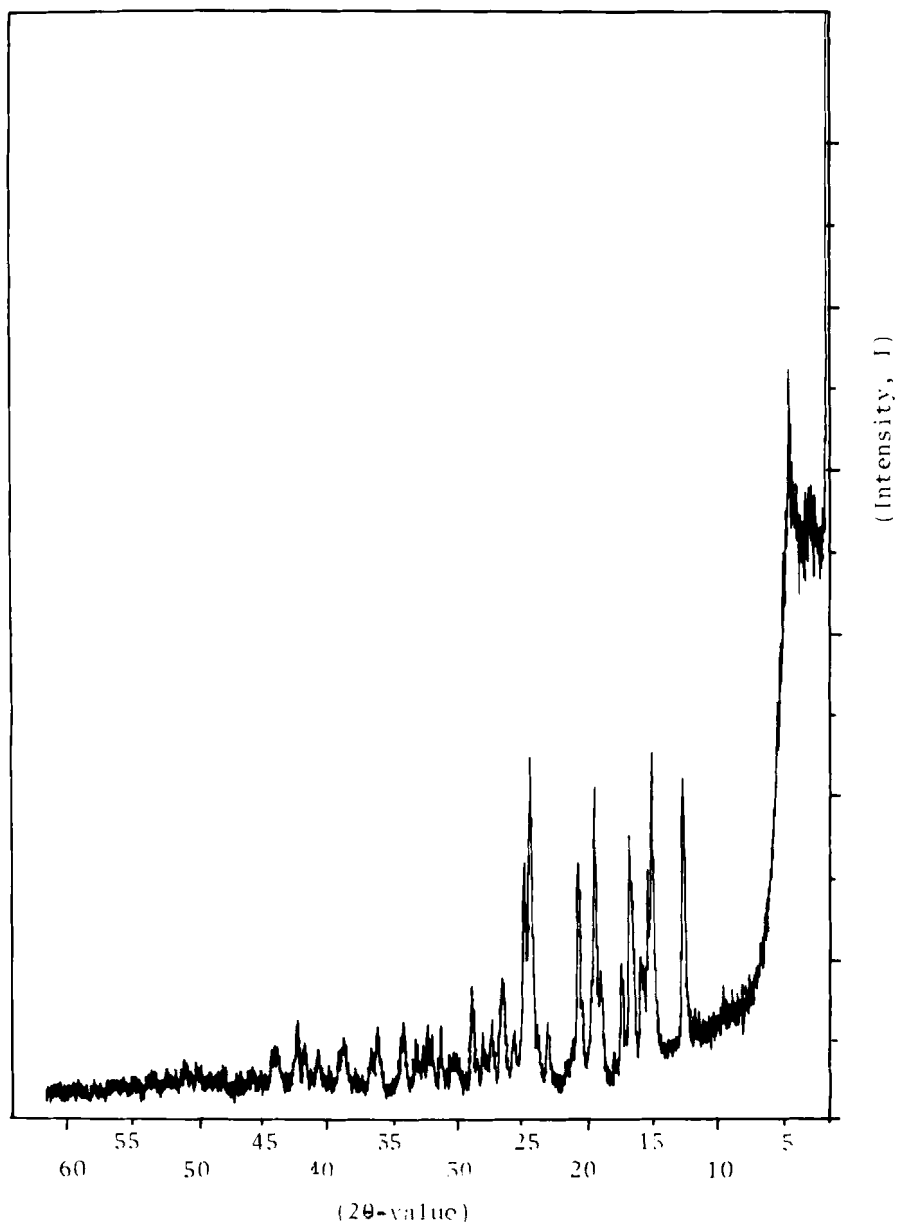


Fig. 4 : Characteristic principal lines of the X-ray powder diffraction of thioridazine hydrochloride.

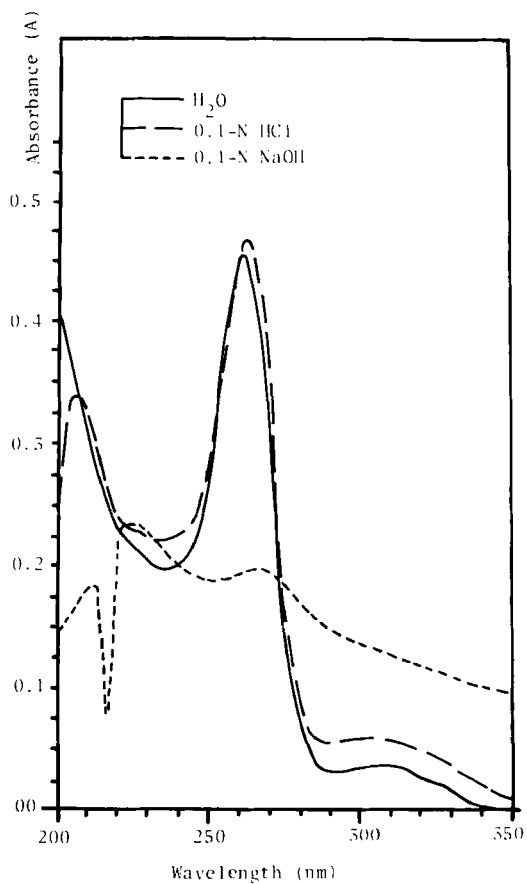


Fig. 5 : The UV (350-200 nm) scanning of 5  $\mu\text{g}.\text{ml}^{-1}$  solutions of thioridazine hydrochloride in water, 0.1-N HCl NaOH

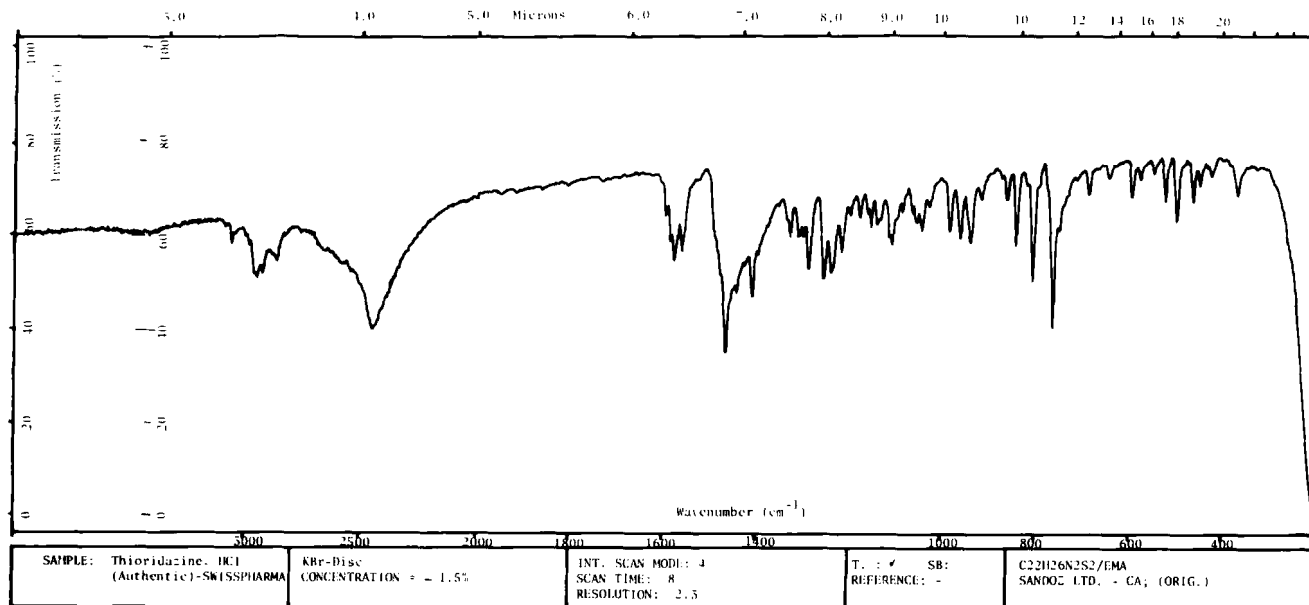


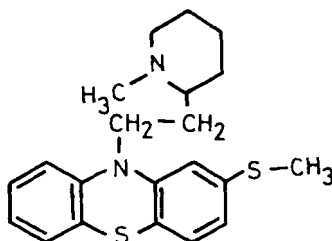
Fig. 6 : The IR-Spectrum of Thioridazine Hydrochloride in KBr.

Table 3: The IR-characteristics of thioridazine hydrochloride.

Frequency, $\text{cm}^{-1}$	Group assignment	Remarks
2960-2880 (w)	$>\text{CH}_2-\text{CH}_3$ , CH stretching	
2480 (m)	$^+\text{HN}\leq$ , stretching	broad due to pre-
1595-1580 (m)	$^+\text{HN}\leq$ , ,	sence of overtone
1460-1410 (s)	$\text{CH}_2$ , $-\text{CH}_3$ , CH-deformation	bands.
1330-1220 (m)	S- $\text{CH}_3$ , S-C stretching.	
760 (s)	$\text{CH}_2$ , rocking.	

3.83 Mass Spectrometry (MS)

The mass spectrum of thioridazine is illustrated in Figure 7, where a base peak appears at  $m/e$  98 due to  $\text{C}_6\text{H}_{12}\text{N}$  i.e., N-methyl piperidyl group. The mass spectrum of thioridazine as the free base, or as the hydrochloride salt, is said to provide a sensitive and specific mean for the identification and quantification in pharmaceutical formulations or biological materials. The mass fragmentation pattern for thioridazine is shown below, the numbers in parentheses representing relative intensities.

Table 4: Mass fragmentation of Thioridazine

$[\text{C}_{21}\text{H}_{26}\text{N}_2\text{S}_2 \quad m/e : 370.5 (34\%)]$


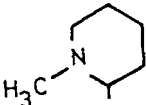
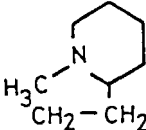
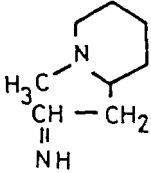
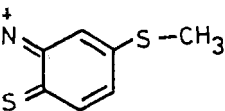
Emperical Structure	Mass/charge Ratio (m/e)	[Io/I (%) ]	Fragment ion
$C_2H_4N$	42	9	$CH_2=CH-NH$
$CH_3S$	47	2	$CH_3S$
$C_6H_4$	76	22	
$C_6H_{12}N$	98	100	
$C_8H_{16}N$	126	16.4	
$C_8H_{16}N_2$	140	3.1	
$C_7H_6NS_2$	167	4	

table 4 contd...

$C_8H_{11}NS_2$	185	19	
$C_{13}H_9NS$	211	12	
$C_{14}H_{12}NS$	226	12	
$C_{13}H_{10}NS$	244	10.9	
$C_{15}H_{13}NS_2$	271	3.1	
$C_{20}H_{23}N_2S$	323	2.1	

### 3.84 Nuclear Magnetic Resonance (NMR)

Both the proton nuclear magnetic resonance ( $^1H$ -NMR) and Carbon nuclear magnetic resonance ( $^{13}C$ -NMR) of thioridazine hydrochloride have been run on the same solution of thioridazine hydrochloride sample dissolved in  $CDCl_3$ .



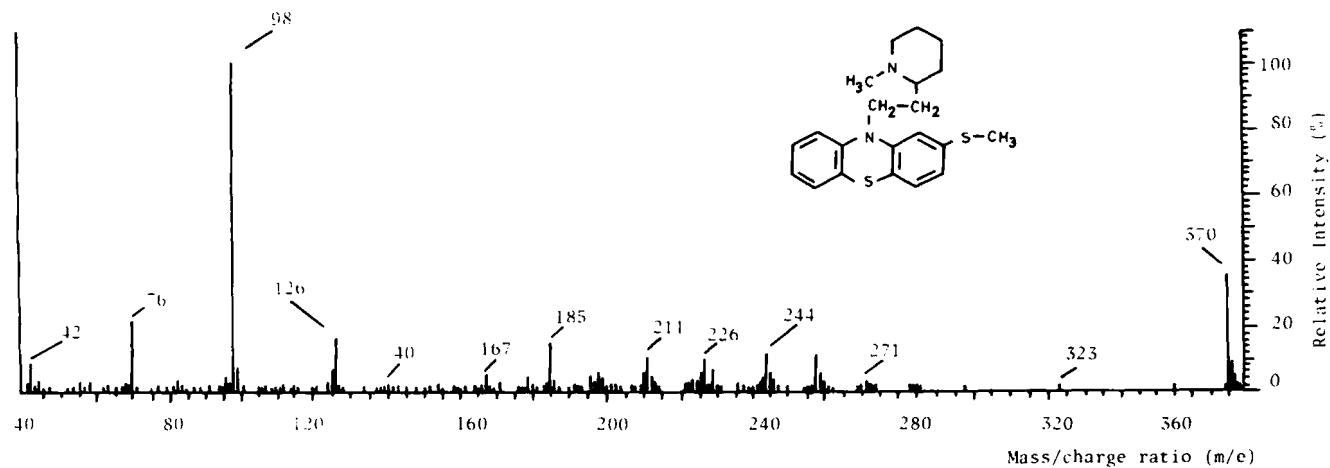
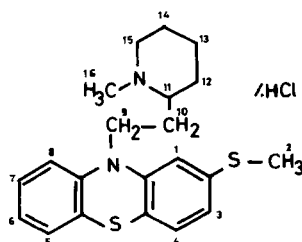


Fig. 7 : The MS-Spectrum of Thioridazine Base.

3.841  $^1\text{H-NMR}$  Spectrum

The 200 MHz proton magnetic resonance spectrum of thioridazine hydrochloride is given in Figure 8 while Table 5 summarizes the chemical shift and spectral assignments of the protons of thioridazine and its hydrochloride salt. The running of the spectra was undertaken in  $\text{CDCl}_3$  and TMS as the internal standard on a Varian XL-200 Spectrometer at ambient temperature.

Table 5 : Chemical shifts and spectral assignments of  $^1\text{H-NMR}$  of thioridazine base and its hydrochloride salt.



<u>Drug</u>	<u>Proton Position (Nr)</u>	<u>(ppm, TMS)</u>	<u>Multiplicity</u>
<u>Thioridazine</u>	aromatic; 1,3-8(7)	7.30-6.70	m
	$\text{CH}_3\text{N}$ & $\text{CH}_3\text{S}$ ; 2,16(6)	2.50-2.30	s
	$\text{CH}_2$ ; 11-15 (9)	1.90-1.30	m
	$\text{CH}_2$ ; 10(2)	3.90-3.50	m
	; 9(2)	4.40-4.10	m
<u>Thioridazine</u>	aromatic, 1,3-8(7)	7.20-6.80	m
<u>hydrochloride.</u>	$\text{CH}_3\text{N}$ & $\text{CH}_3\text{S}$ ; 2,16(6)	2.49-2.46	s
	$\text{CH}_2$ ; 11-15(9)	2.30-1.20	m
	$\text{CH}_2$ ; 10(2)	3.50-2.80	m
	; 9(2)	4.20-3.80	m
	$^+\text{NH}(1)$	11.78	s, broad

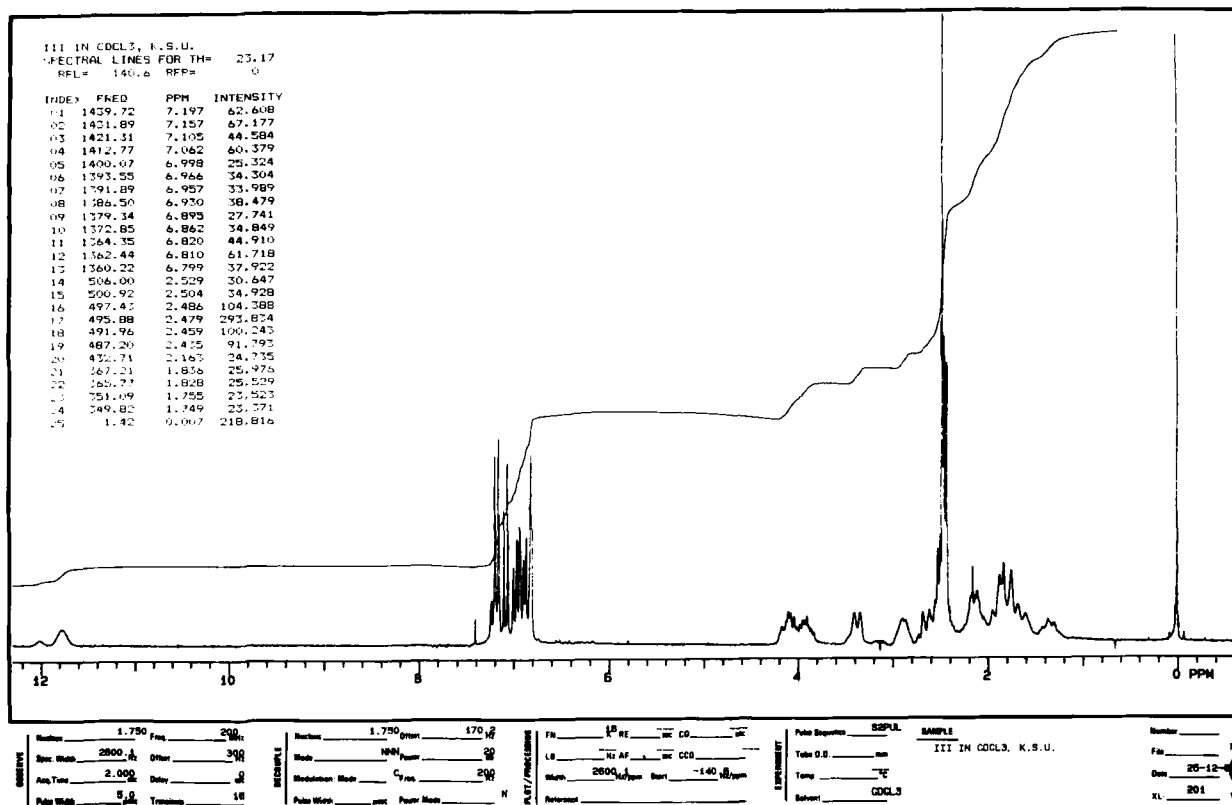
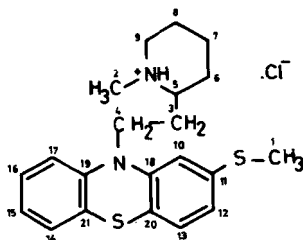


Fig. 8 : The 200 MHz <sup>1</sup>H-NMR Spectrum of Thioridazine Hydrochloride.

### 3.842 $^{13}\text{C}$ -NMR Spectrum

The  $^{13}\text{C}$ -nuclear magnetic resonance spectrum of thioridazine hydrochloride was obtained in  $\text{CDCl}_3$  at ambient temperature using TMS as the internal standard on a Varian XL-200 Spectrometer. The chemical shifts, multiplicities and spectral assignments are given in Table 6, while Figure 9 shows the obtained  $^{13}\text{C}$ -NMR spectrum. The DEPT and APT spectra of thioridazine hydrochloride are given in Figures 10 and 11 respectively.

Table 6: Chemical shifts and spectral assignments of  $^{13}\text{C}$ -NMR of thioridazine and thioridazine hydrochloride.



<u>Carbon position</u>	<u>Assignment†</u>	<u>Chemical shift (<math>\delta</math>, ppm)</u>
C <sub>1</sub>	S-CH <sub>3</sub>	16.08
C <sub>2</sub>	N-CH <sub>3</sub>	40.90
C <sub>3</sub>	-CH <sub>2</sub> -	22.37 <sup>a</sup>
C <sub>4</sub>	N-CH <sub>2</sub> -	56.77
C <sub>5</sub>	-CH-	63.60
C <sub>6</sub>	(CH <sub>2</sub> )	22.97 <sup>a</sup>
C <sub>7</sub>	(CH <sub>2</sub> )	28.51 <sup>*</sup>
C <sub>8</sub>	(CH <sub>2</sub> )	27.68 <sup>*</sup>
C <sub>9</sub>	N-CH <sub>2</sub> -	43.19

table 6 contd.

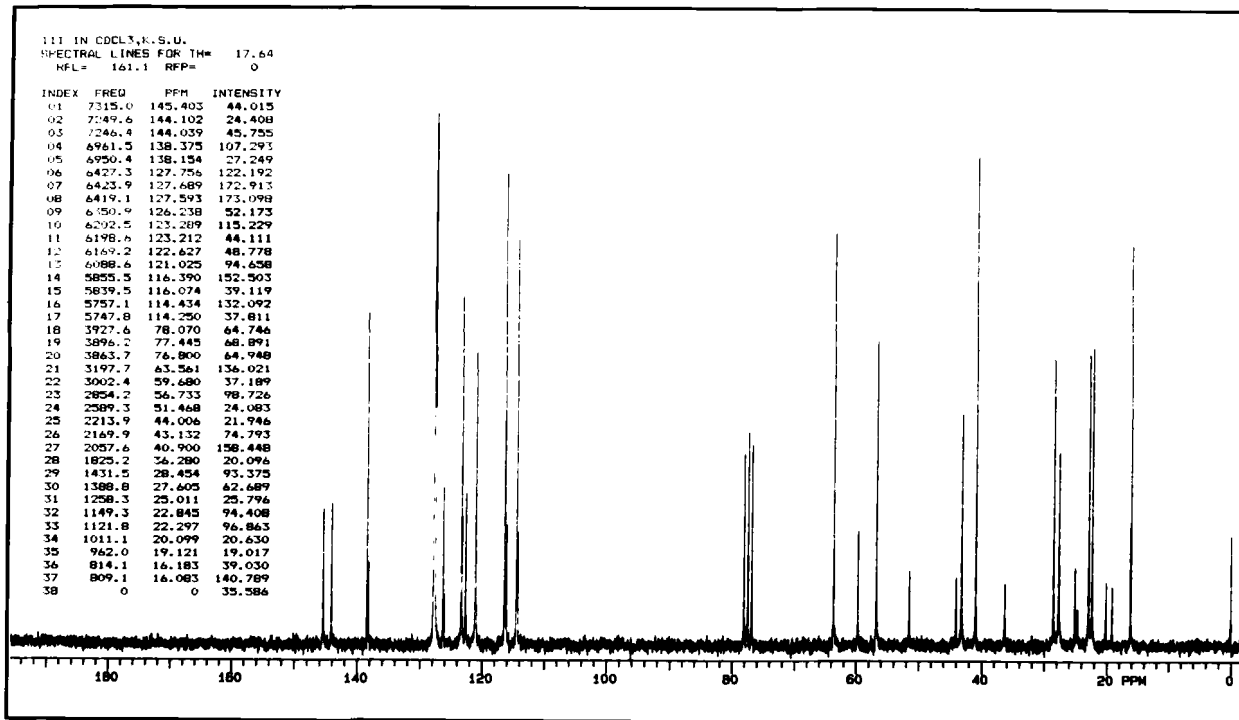
PRINTED IN U.S.A.

PART 10 807306

CHART XL SERIES

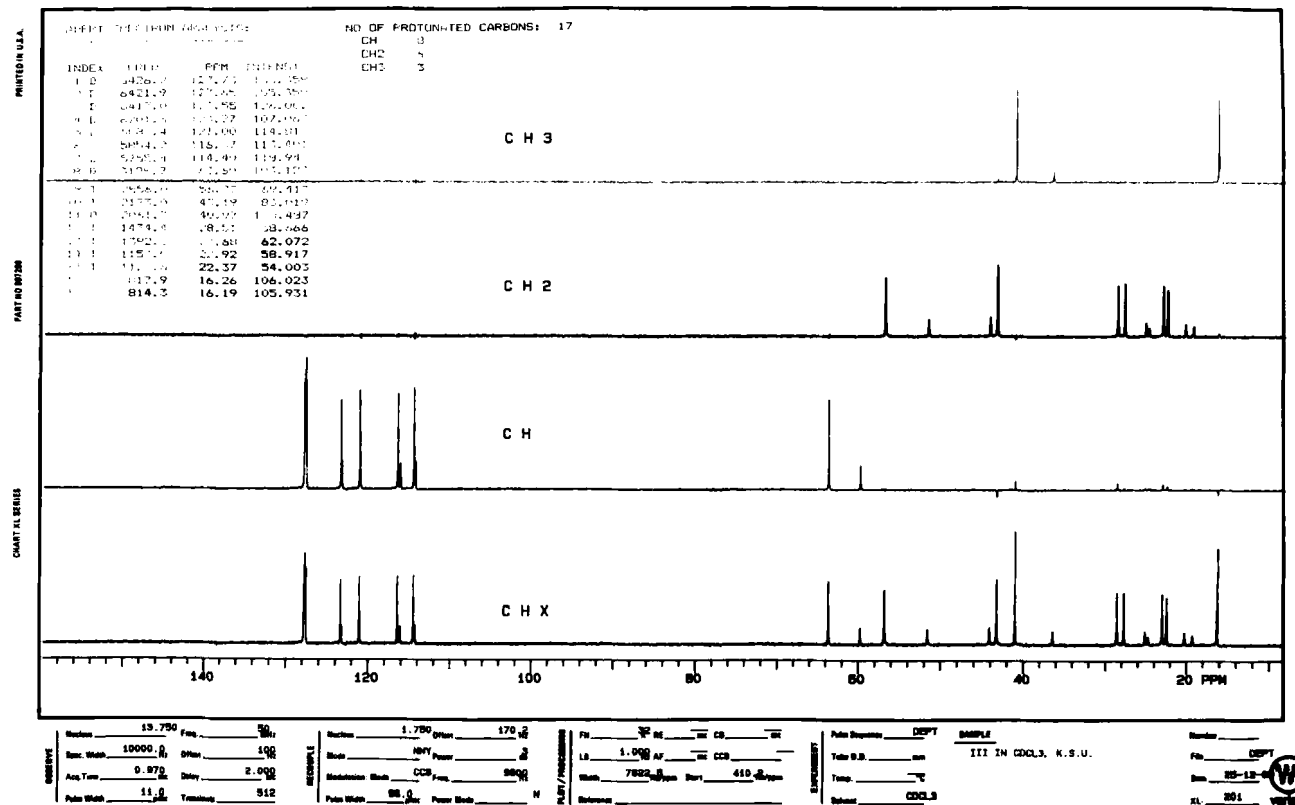
111 IN CDCL<sub>3</sub>, K.S.U.  
SPECTRAL LINES FOR THE 17.64  
MFL= 161.1 RFP= 0

INDEX	FREQ	PPM	INTENSITY
01	7315.0	145.403	44.015
02	7349.6	144.102	24.408
03	7346.4	144.039	45.755
04	6961.5	138.375	107.293
05	6950.4	138.154	27.249
06	6427.3	127.756	122.192
07	6423.9	127.689	172.913
08	6419.1	127.593	173.098
09	6350.9	126.238	52.173
10	6292.5	123.289	115.229
11	6198.6	123.212	44.111
12	6169.2	122.627	48.778
13	6098.6	121.025	94.658
14	5855.5	116.390	152.503
15	5839.5	116.074	39.119
16	5757.1	114.434	132.092
17	5747.8	114.250	37.811
18	3927.6	78.070	64.746
19	3896.2	77.445	60.891
20	3863.7	76.800	64.948
21	3197.7	63.561	136.021
22	3002.4	59.680	37.189
23	2854.2	56.733	98.726
24	2589.3	51.468	24.083
25	2213.9	44.006	21.946
26	2169.9	43.132	74.793
27	2057.6	40.900	158.448
28	1825.2	36.280	20.096
29	1431.5	28.454	93.375
30	1388.8	27.605	62.689
31	1258.3	25.011	25.796
32	1149.3	22.845	94.408
33	1121.8	22.297	96.863
34	1011.1	20.099	20.630
35	962.0	19.121	19.017
36	814.1	16.183	39.030
37	809.1	16.083	140.789
38	0	0	35.584



Receiver: 15.750 Spec. Width: 10000 Acq. Time: 0.970 Pulse Width: 5.0	Freq: 50 Mode: VVY Date: 8 Transmitter: 980	Receiver: 1.750 Mode: VVY Date: 8 Transmitter: 980	Freq: 170.0 Mode: VVY Date: 8 Transmitter: 980	Pulse Sequence: SSPL Tube O.S.: 0.0 Temp: 25.0 Solvent: CDCL <sub>3</sub>	Sample: 111 IN CDCL <sub>3</sub> , K.S.U. Date: 85-12 XL: 801
--	--	---	---	--	---

Fig. 9 : The 200 MHz <sup>13</sup>C Spectrum of Thioridazine Hydrochloride.



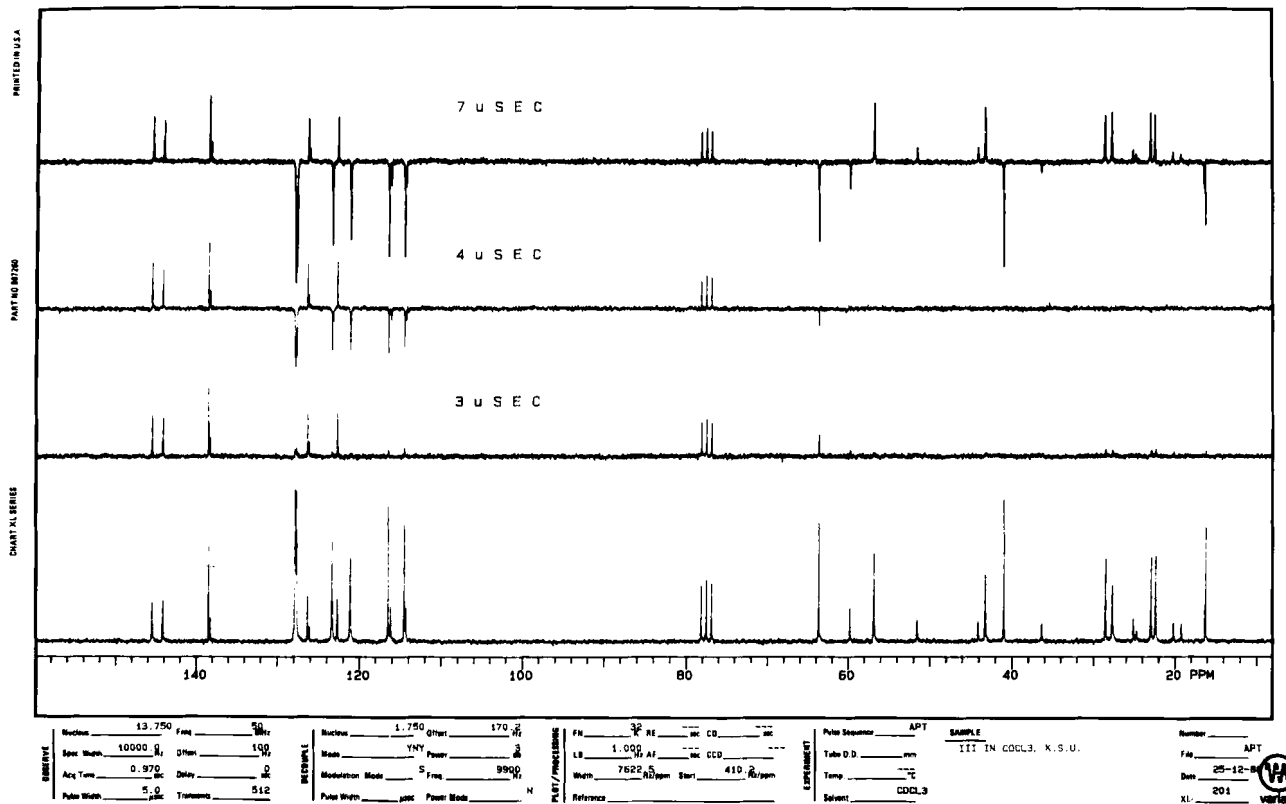


Fig. 11 : The APT Spectra of Thioridazine Hydrochloride.

C <sub>10</sub>	-CH-	114.40 <sup>+</sup>
C <sub>11</sub>	C-aromatic	138.15 <sup>°</sup>
C <sub>12</sub>	CH-aromatic	116.37 <sup>+</sup>
C <sub>13</sub>	CH - ,,	121.00
C <sub>14</sub>	CH - ,,	123.27
C <sub>15</sub>	CH - ,,	)
C <sub>16</sub>	CH - ,,	)121.55-127.73
C <sub>17</sub>	CH - ,,	)
C <sub>18</sub>	C-aromatic	145.40 <sup>x</sup>
C <sub>19</sub>	CH - ,,	144.10 <sup>x</sup>
C <sub>20</sub>	CH - ,,	)
C <sub>21</sub>	CH - ,,	)138.38 <sup>*</sup>

---

\* Carbon-atom assignment was considered by comparing the tabulated chemical shift after calculation according to the individual bonding of each atom. (11).

<sup>°</sup>, <sup>\*</sup>, <sup>+</sup>, <sup>x</sup> indicate possibility of interchange.

#### 4. SYNTHESIS

There are different synthetic methods for preparation of thioridazine starting from various materials.

##### 4.1 Manufacturing (12)

N-(m-methylmercapto-phenyl)-aniline is prepared by condensing m-methyl mercapto-aniline with the potassium salt of o-chloro-benzoic acid and decarboxylating the resultant N(m-methylmercapto-phenyl)-anthranilic acid by heating, and then distilling. 9.87 Parts of N-(m-methylmercapto-phenyl)-aniline are heated with 2.93 parts of sulfur and 0.15 parts of powdered iodine for 15 minutes in a bath at about 160°C. Upon termination of the ensuring evolution of hydrogen sulfide, animal charcoal is added to the reaction mixture and recrystallization carried out first from 40 parts of chlorobenzene and then from 25 to 30 parts benzene at the boiling temperature, 3-methylmercapto-phenothiazine is obtained.

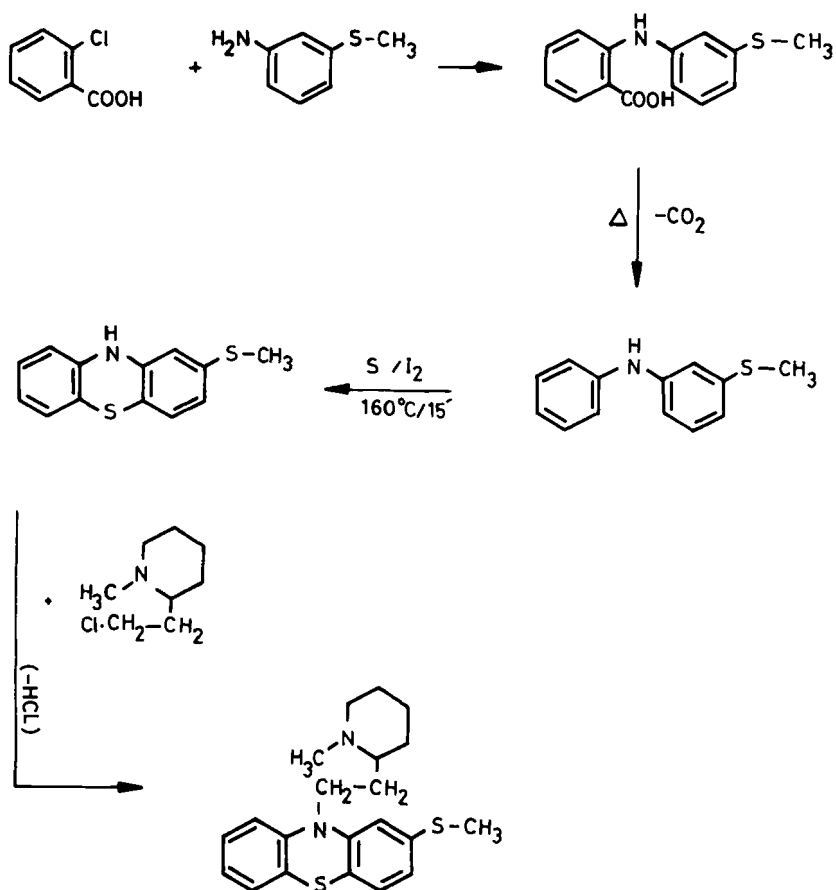
17.82 parts of 2-methylmercapto-phenothiazine, 3.4 parts of finely pulverized sodamide and 80 parts of absolute xylene are heated to boiling for 2 hrs at a bath temperature of 180°C under a reflux condenser and while stirring the reaction mixture. Without interrupting the heating, a solution of 13.2 parts of 2-(N-methyl-



piperidyl-2')-1-chloro-ethane in 40 parts of absolute xylene is then added dropwise in the course of 1-1/2 hr. After further heating for 3 hrs., the reaction mixture is cooled and, after the addition of 5 parts of ammonium chloride, is shaken 3 times with water. The xylene solution is extracted once with 35 parts of 3N acetic acid and then 3 times, each time with 15 parts of acetic acid, after which the acetic acid extract is washed with 60 parts of ether and is then made phenolphthalein-alkaline by means of 25 parts of concentrated aqueous caustic soda solution.

The precipitated oily base is taken up in a total of 100 parts of benzene. The benzene layer, dried over  $K_2CO_3$  is filtered and then evaporated under reduced pressure. The residue from evaporation is distilled in a high vacuum; after separating a preliminary distillate which passes over up to  $228^{\circ}C$  under a pressure of 0.92 mm Hg. the principal fraction, 2-methylmercapto-10-[2'-(N-methyl-piperidyl-2'')-ethyl-1]-phenothiazine, which distills over at  $228^{\circ}C$  to  $232^{\circ}C$  under the mentioned pressure, is collected.

## SCHEME 1

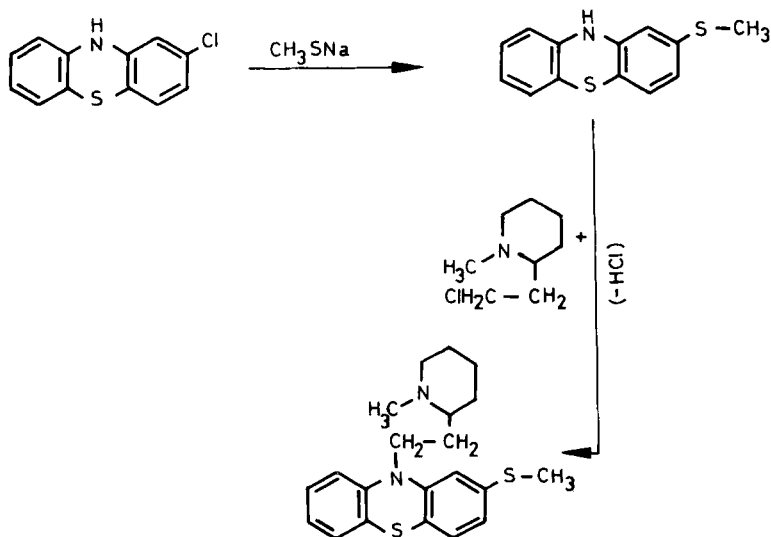


Scheme 1 illustrates the manufacture of thioridazine starting from m-methyl-mercapto-aniline (13).

#### 4.2 Partial Synthesis

2-chlorophenothiazine reacts with methylthio-sodium to yield 2-methylmercapto-phenothiazine, which is condensed with 2-(N-methyl-piperidyl-2')-1-chloro-ethane with the aid of dehydrochlorating agent such as sodamide (5,12). 2-(N-Methyl-2-piperidyl) ethanol (31 parts) in chloroform is treated with HCl at 10°C and 59 parts  $\text{SOCl}_2$  is added. Heating at 70°C is continued for 2 hours, make alkaline with NaOH and extract with ethanol to give 2-(N-methyl-2-piperidyl-2')-1-chloroethane. 2-(Methylmercapto) phenothiazine, 16.7 parts and 2[N-methyl-2-piperidyl -2'] 1-chloroethane, 10 parts, are heated with 2.3 parts sodamide in 100 parts xylene and refluxed for 3 hrs to yield thioridazine (14). Scheme 2 illustrates the synthetic pathway of thioridazine starting from 2-methyl-mercaptophenothiazine.

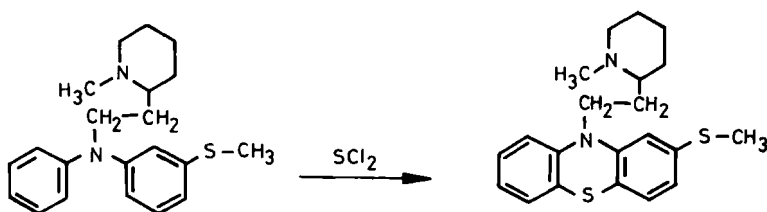
SCHEME 2



### 4.3 Cyclization

Bourquin *et al* (14) described a synthetic pathway for obtaining thioridazine by cyclizing the N-(m-methylmercaptophenyl), N-phenyl, N-(2-(N-methyl piperidyl)-1-ethylamine with sulfur dichloride. Scheme 3 demonstrates the mentioned cyclization to thioridazine.

### SCHEME 3



## 5. PHARMACOKINETICS

### 5.1 Absorption

Thioridazine like other phenothiazines is generally well absorbed from the GI tract and from parenteral routes, however, absorption may be erratic, particularly following oral intake. Many interindividual variations in peak plasma contractions may be attributed to genetic differences in the rate of metabolism of the drug during absorption in the GI-mucosa and pass through the liver (5,8). Following oral administration of thioridazine, the drug is detectable in serum after 1 hr post administration (16). Following multiple daily dosing, accumulation of the drug occurs within 3 to 4 days and serum levels may be maintained for 100 to 120 hrs after withdrawal of the drug (17).

### 5.2 Distribution

Thioridazine and its metabolites are distributed into most body tissues and fluids, with high concentrations being distributed into the cerebrospinal fluid (CSF), lungs, liver, kidneys and spleen. In the same mode of distribution of phenothiazines, thioridazine is highly bound to plasma proteins. The drug can readily cross the placenta. It is not known if the drug is distributed

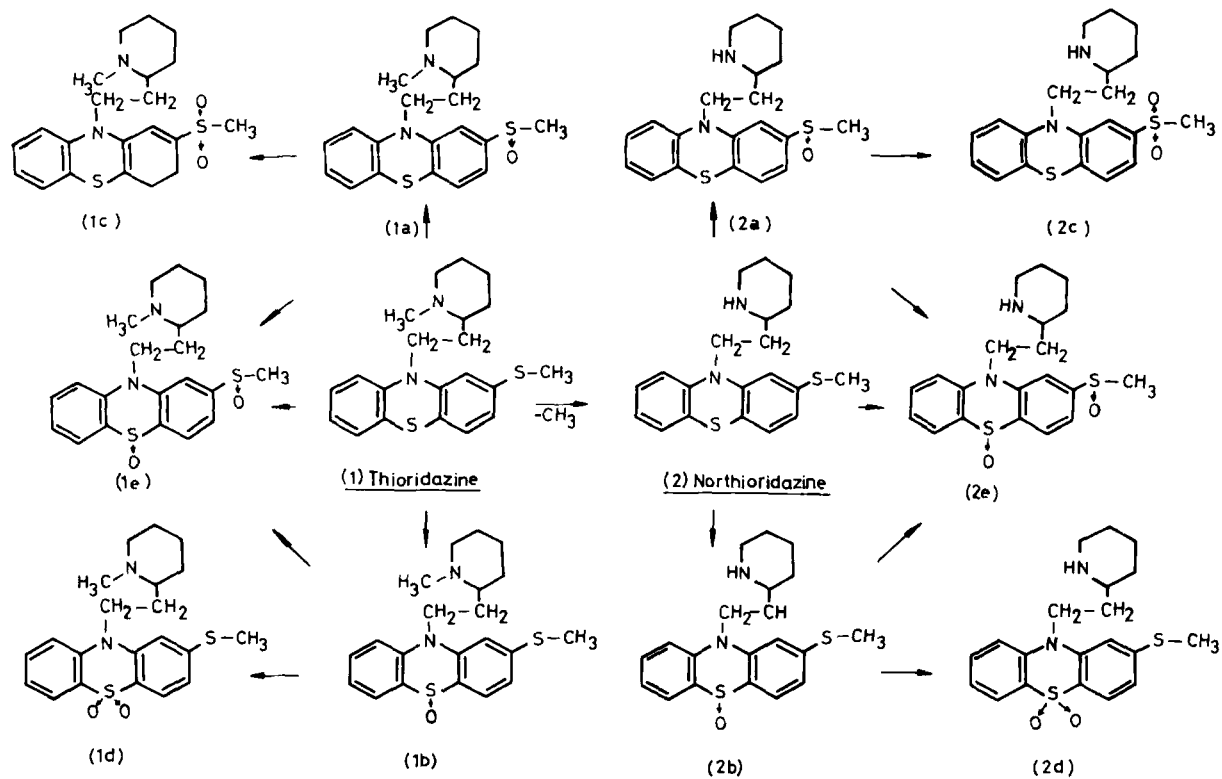
into milk; however, the size of the molecules and their ability to cross the blood-brain barrier suggest that the drug would be distributed into milk (8). Mean total concentration of thioridazine in the CSF was 19.4 n moles in patients treated with 40-1000 mg.day<sup>-1</sup>.

### 5.3 Biotransformation

Thioridazine, like other phenothiazines, undergoes a series of metabolic transformations in the organism. Several non-conjugated metabolites were identified, when radioactively-labelled thioridazine was given to rats (18). Among those metabolites are side-chain sulfoxide [1a], ring sulfoxide [1b], side-chain sulfones [1c], ring sulfones [1d], disulfoxides [1e] and disulfones as well as the corresponding demethylated compounds [2a-e] (18,19). Scheme 4 demonstrates the formation pathways of different metabolites of thioridazine.

It is known that at least two of the metabolites of thioridazine, namely thioridazine-2-sulfoxide, i.e. mesoridazine, and thioridazine-2-sulfone, i.e., sulforidazine, are pharmacologically active (20-23). Buyze et al (24) described different metabolic possibilities for thioridazine in human, but gave no details as to how much the metabolites were identified and quantified. Mårtensson et al (19) described analytical procedures for extraction, identification and determination of non-conjugated thioridazines metabolites in serum and urine of psychiatric patients undergoing long time thioridazine treatment. The concentration of unchanged thioridazine in serum is roughly equal to that of its side-chain sulfoxide, i.e. mesoridazine, which is known to be a psychoactive drug in itself (19). The clinical effect of thioridazine may be the result of a total effect of a variety of substances such as one or several metabolites. Using rabbit striatal slices in an in-vitro technique, Niedzwieck et al (25) found that both clinically active metabolites mesoridazine and sulforidazine are significantly more potent in blocking dopamine receptors than the parent compound. Serum concentrations of a side-chain sulfoxide and sulfone correlate with scores for side effects such as dry mouth and tremor but not for drowsiness and nasal congestion (26).

# SCHEME 4



## 5.4 Drug Concentration Levels

### 5.41 Therapeutic Plasma Levels

Axelsson and Mårtensson (26) studied the serum concentration and elimination from serum of thioridazine in 169 psychiatric patients, 103 women and 66 men selected from 200 in-patients who had been treated with thioridazine for at least 8 days. The evening serum concentration was varying from 0.05 to 2.82  $\mu\text{g}.\text{ml}^{-1}$ , while the morning concentration between traces and 2.17  $\mu\text{g}.\text{ml}^{-1}$ . The serum concentration reaches a plateau within a week. Comparative evaluation of the serum concentrations of thioridazine and its active metabolites sulforidazine and mesoridazine was undertaken by using radioreceptor assay and HPLC (27-30).

### 5.42 Toxic Plasma Levels

Serum concentrations ranging from 2.4 to 11.8  $\mu\text{g}.\text{ml}^{-1}$  have been recorded during non-fatal intoxications with thioridazine (31,33). Blood levels of 0.8 to 13  $\mu\text{g}.\text{ml}^{-1}$  were reported for fatal overdose (33).

## 5.5 Elimination

### 5.51 Plasma Protein Binding

In 48 patients treated with thioridazine, the mean amount not bound to serum proteins was 0.15%, that of the side-chain sulfoxide 1.66%, side-chain sulfone 1.17%, and ring sulfoxide 1.70% (34).

### 5.52 Half-Life ( $T_{1/2}$ )

The mean serum  $T_{1/2}$  in 20 patients treated with thioridazine only was 16.1 hrs, but 17.1 hrs in patients who received also additional medications (35). The  $T_{1/2}$ -value is recorded also to be 24 hrs in some studies (36). The interindividual variation of the elimination of thioridazine from serum was thus of the same magnitude as the variation of the serum concentration. In elderly patients, a decreasing ability to eliminate thioridazine, i.e.,

diurenal decrease, was observed (35); this means that a given dose of thioridazine will give a higher serum concentration in elderly patients than in younger ones.

#### 5.53 Effect of Other Medications on Serum level and Elimination

Axelsson and Mårtensson (35) observed that alcoholics eliminated thioridazine faster than the other patients even when were receiving increasing doses. This was explained by interindividual differences in metabolism of alcoholics, whom liver enzymes may be induced by alcohol. It was reported that the addition of propranolol, a lipid-soluble B-adrenergic receptor antagonist, commonly prescribed for hypertension, to the pharmacotherapy of patients receiving thioridazine increased the serum levels of thioridazine into the potentially toxic range (37-39). It is known that the adverse reactions are more likely to be related to the concentration of thioridazine in the body than to the dose ingested, the clinician should alert to initiate the corrective measures in each individual case.

#### 5.54 Total-Plasma and Unbound Plasma Concentrations

Neuroleptic drug, e.g. thioridazine, concentrations at the receptor sites are likely to be reflected more clearly by the unbound than by the total plasma concentrations. The red blood cells (RBC) concentrations showed the best correlation to the unbound plasma values, i.e., may be more accurate than the total plasma concentration of the drug. The determined unbound plasma concentrations of the thioridazine were more accurate image. The total plasma concentrations, but neither the unbound plasma nor the RBC concentrations, were significantly correlated to the concentrations of the drug-binding protein  $\alpha_1$ -acid glycoprotein. Radioreceptor assay values were also strongly correlated to the weighed serum of the total and unbound plasma concentrations of thioridazine and its metabolites (37).



### 5.55 Excretion

Thioridazine is extensively metabolized, principally in the liver via hydroxylation, oxidation, demethylation, sulfoxide formation and conjugation with glucuronic acid; metabolic alterations in the side-chain may also occur. At least two metabolites of thioridazine are pharmacologically active; while most of them are inactive. Thioridazine and its metabolites are excreted in urine and feaces; the excretory patterns have not been fully characterized. The drug is excreted in feaces via biliary elimination, principally as metabolites, and also appears to undergo enterohepatic circulation. Certain metabolites and only small amounts of the unchanged drug have been detected in urine in some patients for upto 6 months following stopping of therapy with the drug (40,41).

## 6. THERAPEUTIC CATEGORATION

### 6.1 Pharmacology

Like the other phenothiazines antipsychotic agents, thioridazine has also been described as neuroleptic agents because of its activity in inducing the neuroleptic syndrome (i.e., depressed initiative, decreased effect, disinterest in surroundings, supression of complex behavior and spontaneous movements, decreased agressiveness and impulsivity, extrapyramidal action).

Phenothiazine, the structural prototype of the phenothiazines, is not more in use as urinary tract antiseptic due to its toxicity, but still used as an anthelmintic in veterinary medicine and as an insecticide (42). The development of phenothiazines as psychopharmacological agents resulted from the observed sedation activity of certain antihistaminic phenothiazine compounds. In an attempt to enhance the sedative effects of such group of drugs, some analogues were synthesized.

The pharmacology of thioridazine as all other phenothiazines is complex, and due to its activity on the central and autonomic nervous systems, the drug affects many different sites in the body.

### 6.11 Effects on Nervous System

Thioridazine acts principally in the CNS at the subcortical levels of the reticular formation, hypothalamus and limbic system, without producing substantial cortical depression. The drug acts also in the basal ganglia, exhibiting extrapyramidal effects.

The real mechanism(s) of action of thioridazine, including antipsychotic one, has not been determined, but may be related principally to its antidopaminergic effects. There is evidence to indicate that thioridazine and other phenothiazine's antipsychotic antagonize dopamine-mediated neurotransmission at the synapses. Thioridazine may block postsynaptic dopamine receptor sites. However, it is not sure whether the antipsychotic effect of thioridazine is definitely related to their antidopaminergic effects. Thioridazine also has principal and/or central antagonistic activity against  $\alpha$ -adrenergic, serotonergic, histamine ( $H_1$ -receptors), and muscarinic receptors. The effects of the drug on the autonomic nervous system are complex and unpredictable since the drug exerts varying degrees of  $\alpha$ -adrenergic blocking, muscarinic blocking, & adrenergic activity. It has also been suggested that the drug's effects on dopamine are probably most important, but the drug's effects on the other amines, such as  $\gamma$ -aminobutyric acid (GABA), or peptides, such as substance P, endorphins, may contribute to the antipsychotic effects of thioridazine (42).

Like other phenothiazines thioridazine produces varying degrees of sedation without hypnosis or anesthesia in normal and psychotic patients; however, it potentiates the CNS depressant activities of sedatives, hypnotics and anesthetics. Thioridazine increases total sleep time, normalizes sleep disturbance in psychotic patients, and decreases REM-sleep. The drug may cause EEG-changes, including a slowing of the EEG pattern with increase in  $\theta$ - and  $\Delta$ -wave responses and some decrease in fast-wave and  $\alpha$ -wave activity (42).

On the weight basis, thioridazine is about as potent as chlorpromazine, but has strong anticholinergic and sedative effects and weak extrapyramidal effects. Thioridazine has little antiemetic activity, which would be mediated via a direct effect on the medullary chemoreceptor trigger zone (CTZ), apparently by blocking dopamine receptors in the CTZ. Thioridazine inhibits the central and peripheral effects of apomorphine and ergot alkaloids (42).

#### 6.12 Effects on Cardiovascular System (CVS)

Thioridazine has direct and indirect actions on the heart and vasculature making the cardiovascular effect complex. The drug inhibits peripheral  $\alpha$ -adrenergic blocking activity and causes vasodilation leading to orthostatic hypotension. The drug may increase the coronary blood flow as a result of increased heart rate. Transient antiarrhythmic effects have been observed in some patients at higher dosages. This may result from either a direct quinidine-like properties or probable local anaesthetic effect of the drug.

#### 6.13 Effects on Endocrines

Thioridazine may induce secretion of prolactin from the anterior pituitary by inhibiting dopamine receptors in the pituitary and hypothalamus during long-term administration. Prolactin secretion may be accompanied also with amenorrhea, gynecomastia and impotence. Decreases of urinary concentrations of gonadotropin progestins may be observed in some patients, and may be vasopressin and corticotropin in some other patients(42).

#### 6.14 Other Effects

Thioridazine may has antiinflammatory and/or antipruritic effects, resulting from antagonism of various mediator substances, such as serotonin and histamine.

## 6.2 Uses

Thioridazine is mostly prescribed for the symptomatic management of psychotic disorders. The drug is also used for the short-term treatment of adults with major depression who have varying degrees of associated anxiety (40).

## 6.3 Drug-Drug Interactions

### 6.31 CNS Depressants

Since thioridazine may be additive with, or may potentiate the action of, other CNS depressants such as opiates or other analgesics, barbiturates or other sedatives, general anaesthetics, or alcohol. Caution should be taken to avoid probable excessive sedation or CNS depression (42).

### 6.32 Lithium

Patients receiving combined therapy of lithium and thioridazine may exert acute encephalopathic syndromes occasionally occurring especially when higher serum lithium concentrations are present. Such patients should be observed for evidence of adverse neurologic effects and treatment should be rapidly discontinued if those signs or symptoms appear (40,43,44).

### 6.33 Metrizamide

The concurrent use of metrizamide with thioridazine, a drug lowers the seizure threshold, an increased risk of seizures can be expected. The manufacturers state that phenothiazines should not be used in patients receiving metrizamide and for the control of metrizamide-induced nausea and vomiting (42,44).

### 6.34 Anticonvulsants

Because of the seizure-threshold lowering effect of thioridazine, dosage adjustment of anticonvulsants may be necessary when they are prescribed with thioridazine. The CNS depressant effects of

thioridazine and other phenothiazines do not potentiate the anticonvulsant activity of anticonvulsants (42).

#### 6.35 Bromocriptine

Robbins et al (45) reported that patient receiving thioridazine and given bromocriptine for a large prolactin secreting pituitary adenoma shows increases of serum prolactin level. Some adverse effects, such as deterioration of visual fields, were resolved after stopping thioridazine. It was concluded that the use of dopamine antagonists such as thioridazine in patients with prolactinoma may interfere with bromocriptine's action, resulting in potentially serious complications.

#### 6.36 Phenytoin

Marcoux (46) observed an increase in self injurious behaviour following initiation of phenytoin therapy in mentally retarded patients receiving thioridazine. Although the clinical observations of Sands et al (47) showed that the alterations of thioridazine's serum concentrations by phenytoin are infrequent.

#### 6.37 Adsorbent antacid and antidiarrheal

Moustafa et al (48) reported about equivalent decrease in bioavailability of thioridazine due to possible interactions with milk, magnesium trisilicate, bismuth subnitrate, kaolin-pectin mixture of aluminium hydroxide-magnesium carbonate mixture. The decrease was not at adsorption rate but in the total plasma concentration.

### 6.4 Toxicology

#### 6.41 Manifestations

Overdoses of thioridazine or other antipsychotic drugs may be expected to produce effects that are extensions of the common adverse reactions; serve extrapyramidal reactions, hypotension, and sedation have been the main effects reported. Also, CNS

depression progressing to coma with areflexia may occur, this can be accompanied by tachycardia, ECG-changes and cardiac arrhythmias, hypothermia, miosis, tremor, muscle twitching, spasm or rigidity, seizures, muscular hypotonia, ileus, dry mouth, difficulty in swallowing or breathing, cyanosis, and respiratory and/or vasomotor collapse, even with abrupt apnea (49-51).

6.42  $LD_{50}$  (orally in rats) =  $995 \pm 39 \text{ mg.kg}^{-1}$  (4,52).

#### 6.43 Treatment

Treatment of thioridazine overdosage involves symptomatic and supportive care. There are no specific antidotes for thioridazine intoxication; however, anticholinergic antiparkinsonian drugs may be useful in management of extra pyramidal reactions accompanied by thioridazine overdosage. The stomach should be emptied by gastric lavage following acute ingestion of thioridazine. If the patient is comatose, having seizures or a dystonic reaction, gastric lavage may be performed with an endotracheal tube with cuff inflated to avoid aspiration of gastric contents. Due to great reduction of GI-motility following overdosage of thioridazine, gastric lavage may be useful even several hours after the drug ingestion. Administration of a saline cathartic may be beneficial in enhancing evacuation of the drug from the GI-tract. Suitable therapy should be instituted if hypotension occurs; ephedrine should be avoided (42). In acute toxicity exchange transfusions may be beneficial, but hemodialysis is of little value for rapid elimination of the drug.

#### 6.5 Cautions

Care should be taken to avoid skin contact with thioridazine or thioridazine hydrochloride, since contact dermatitis has been observed. Thioridazine appeared to block use of feedback information, this observation was obtained from an evaluation of judgment's performance in some schizophrenic patients (53).

## 6.6 Administration and Dosage

### 6.61 Administration

Thioridazine and thioridazine hydrochloride are administered orally, but when thioridazine hydrochloride oral concentration solution is used, the dose should be diluted (with water or fruit juice) just before administration (40,54).

### 6.62 Dosage

Dosage of thioridazine and thioridazine hydrochloride is expressed in terms of the hydrochloride salt. Dosage must be carefully adjusted according to individual requirements and response using the lowest possible effective dosage. Dosage should be increased more gradually in debilitated or geriatric patients.

For the symptomatic control of psychotic disorders, the usual initial adult dosage of thioridazine is 50-100 mg 3 times daily. Recommended dosages greater than 300 mg daily be reserved for adults with severe neuropsychiatric conditions. Dosages up to 800 mg daily given in 2-4 divided doses may be required in hospitalized, or severely psychotic adults. Dosage during prolonged maintenance therapy with thioridazine should be kept at the lowest effective level; once an adequate response has been obtained, dosage should be gradually reduced and subsequently adjusted according to the patient's therapeutic response and tolerance. Because of the risk of adverse reactions associated with cumulative effects of phenothiazines, patients with a history of long-term therapy with thioridazine and/or other antipsychotic agents should be evaluated periodically to determine whether drug therapy could be discontinued.

For the short-term treatment of adults with major depression who also have varying degrees of associated anxiety, or for the symptomatic management of agitation, anxiety, depressed mood, tension, sleep disturbances, and fears in geriatric patients, the usual initial dosage of thioridazine

is 25 mg 3 times daily. Dosage ranges from 20-200 mg daily in these patients, depending on the severity of the condition.

Thioridazine dosage range from 0.5-3 mg.kg<sup>-1</sup> daily for children 2-12 years of age. Dosage for younger than 2 years of age have not been established. The usual initial dosage of thioridazine is 10 mg 2 or 3 times daily. Dosage may be gradually increased until optimum therapeutic effect is obtained. For the control of hospitalized, severely disturbed, or psychotic children 2-12 years of age, the usual dosage of thioridazine is 25 mg 2 or 3 times daily. Dosage may be gradually increased until optimum therapeutic effect is obtained. Dosage for children should not exceed 3 mg.kg<sup>-1</sup> daily (40).

#### 6.7 Pharmaceutical Preparations

##### Thioridazine

Oral Suspension - equivalent to thioridazine hydrochloride 25 or 100 mg/5 ml. Mellaril-S, Sandoz.

##### Thioridazine Hydrochloride

Oral Solution, concentration - 30 mg/ml [Mellaril Concentrate (with alcohol 3% and parabens), Sandoz; Thioridazine HCl Intensol, Roxane.]

100 mg/ml - Mellari Concentrate (with alcohol 4.2% and parabens), Sandoz; Thioridazine.HCl Intensol, Roxane.

Tablets - 10 mg, 15 mg, 25 mg, 150 mg and 200 mg Mellaril (with parabens and povidone).

Sandoz 50 mg Mellaril, Sandoz. 100 mg Mellaril (with povidone), Sandoz.

Tablets, film-coated

10 mg, 15 mg, 25 mg, 50 mg, 100 mg and 200 mg (available by nonproprietary name) (40).

Other Proprietary Names:

Arg.-Meleril; Belg.-Mellerettes; Canada.-Mellaril, Novoridazine, Thioril; Ger.-Melleretten; Ital.-Mellerette; Neth.-Melleretten; Spain.-Meleril; Swed.-Mallorol; Switz.-Melleretten; USA.-Mellaril., TP21, Sonapax, (Also available as Melleril in Austral., Belg., Denm., Fr., Ger., Ital., Neth., Norw., S. Afr., Switz.) (3,4,8).



### 6.8 Stability

Commercially available thioridazine oral suspension and thioridazine hydrochloride oral concentrate solution should be stored in tight, light-resistant containers at a temperature less than 30°C, preferably between 15-30°C; freezing should be avoided. Thioridazine hydrochloride tablets should be protected from light and stored in well-closed containers at a temperature less than 40°C preferably at 15-30°C (40).

### 6.9 Laboratory Test Interferences

Urinary metabolites of thioridazine may cause the urine to darken and result in false-positive test results for urobilinogen, amylase, uroporphyrins, porphobilinogens, and 5-hydroxyindolacetic acid. False-positive test results for phenylketonuria (PKU) may also occur during phenothiazine use.

False-positive pregnancy test results have reportedly occurred in some patients receiving phenothiazines (42).

## 7. ANALYTICAL METHODS

### 7.1 Qualitative

#### 7.11 Pharmacopoeial Identifications

USP XXI (1) specifies the comparison of the IR-spectrum of the sample with a USP-reference standard of thioridazine base or its HCl-salt, in addition to the test for chloride in case of the salt.

BP 1980 (2) recommends the concordance of the sample with corresponding IR-spectrum of the reference drug substance. Light absorption scanning (350-230 nm) of ethanolic solution of the drug is also recommended beside the blue coloration with sulfuric acid and also the reactions characteristics of chloride in case of the HCl-salt.

#### 7.12 Color Tests

<u>Reagent</u>	<u>Color</u>	<u>Sensitivity</u>	<u>Reference</u>
Sulfuric acid-formaldehyde	Purple-red -- blue green violet (in case of mesoridazine).	0.1 mg	3,55
Ammonium molybdate.	Deep blue -- green	0.1 mg	3,55
Ammonium Vandate	Deep blue -- purple	0.1 mg	3,55
Vitali's test	Blue-purple/purple-brown/yellow brown.	0.25 mg	3,55
Forrest's reagent	Blue Red (in case of mesoridazine).	--	55

### 7.13 Screening Test

<u>Reagent</u>	<u>Color</u>	<u>Specimen</u>	<u>Reference</u>
2% Ferric chloride/ 30% sulfuric acid.	Pale pink deeping to blue.	Urine	55

### 7.14 Micro-crystal Test\*

<u>Reagent</u>	<u>Crystals</u>	<u>Sensitivity</u>	<u>Reference</u>
Gold-cyanide solution.	Branching needles (overnight).	1 in 200	3
Kraut's reagent	Orange-red precipitation	1 in 50	

\* Thioridazine base and its salt give no precipitation with platinic acid, picric acid, zinc chloride, sodium dihydrogen phosphate, mercuric chloride, Marm's and Wagner's reagents.

## 7.15 Chromatography

### 7.151 Zone Electrophoresis

Jokl and Dolejsova (56) studied the electrophoretic behaviour of some phenothiazine anti-psychotics including thioridazine, perphenazine, diethazine, promethazine, fluphenazine, methotri-mepazine, and prochlorperazine. Good separation could be achieved with citric acid, ethylendamine, trisbuffer containing dimethylformamide at different pH-values.

### 7.152 Paper Chromatography (PC)

Solvent Systems : Thioridazine, like other phenothiazines behaves chromatographically in a very similar way to other strong bases. In acidic media they are strongly dissociated; beyond pH 9 they migrate practically in the non-dissociated form. However, thioridazine belongs to that group of substances with a significantly lipophilic nature and therefore the application of current aqueous systems, with lower aliphatic alcohol, did not result in successful separations. The use of systems with a polar organic stationary phase or reversed-phase chromatography was more successful. Due to the instability of thioridazine it is recommended that development in direct daylight must be avoided and development in the dark is preferable.

Good separations are obtained on papers impregnated with 40-50% ethanolic solution of formaldehyde with addition of 5% ammonium formate with a mobile phase of cyclohexane-benzene (90:10, v/v) (57). Similarly good separation are obtained with a reversed-phase system containing a 50% solution of kerosine (b.p. 180-215°C) in light petroleum as the stationary phase and ethanol-water-ammonia (60:38:2, v/v/v) or alternatively (75:23:2, v/v/v) as a mobile phase (57-59). In case of

thioridazine hydrochloride it is possible to impregnate the chromatogram by pipping the paper together with the spotted samples into the kerosine solution. Street (60) recommended 10% glycerol tributyrate (tributyrine) in acetone for impregnation. For development of aqueous solutions of buffer e.g. 2M acetate buffer pH 4.58 was utilized. In order to increase the rate of development the separation can be carried out at 85-95°C, which resulted in a shortening of the developing time by up to 15 min.; in this case the use of thick chromatographic papers proved to be better (61). Many aqueous systems are recommended but the most successful system is the salt solutions of 1N sodium acetate and 1N sodium formate-n-propanol (9:1,v/v) (62). The  $hR_f$  values of thioridazine and mesoridazine are summarized in Table 7.

Table 7: PC  $hR_f$  - values of thioridazine and thioridazine-2-sulfoxide (mesoridazine).

<u>Solvent system</u>	<u><math>hR_f</math> - value*</u>		<u>Reference</u>
	<u>Thioridazine</u>	<u>Mesoridazine</u>	
1N sodium acetate	24	54	62
1N sodium formate-n-propanol (90:10)	40	61	62
Formamide + ammonium formate			
Cyclohexane-benzene (90:10)	43		57
Kerosene/ethanol-water-ammonia (60:38:2).	16		57
Kerosene/ethanol-water-ammonia (75:23:2).	35		58,59
Tributyrin/0.2 M acetate buffer pH 4.58, at 95°C	19		60

$$*hR_f = R_f \times 100$$

Detection: For detection of the drug it is possible to use UV-light, especially in the short region, where fluorescence or absorption can be observed (61). Because of the photooxidation of thioridazine and its hydrochloride salt, it is recommended to record the fluorescence or absorption immediately after taking the chromatogram out of the jar. The color of thioridazine spots can also be influenced by the rests of the solvent system used. According to Mellinger and Keeler (62) the fluorescence is mainly influenced by the chemical structure of the phenothiazines. The main role in this respect is due to the substituent on the C-2 carbon in the phenothiazine ring (61). The substitution by an alkylmercapto-group in thioridazine results in bluish yellow fluorescence or blue fluorescence. On a fluorescent layer of silica gel thioridazine like other phenothiazines appear mostly as quenching spots. As for basic drugs Dragendorff's reagent, or detection with iodoplatinate are the most common chemical methods for detection of thioridazine. Beside these reagents, sulfuric acid in various modifications is usually used, it has the advantage of distinguishing the different phenothiazines by color. It is possible to spray with aqueous 20-50% or 10% ethanolic solutions of sulfuric acid. Sulfoxides react with these reagent more slowly than the original drug (63). Sulfuric acid with anhydrous sodium sulfate (4:1 v/w) (60), and formaldehyde-sulfuric acid (Marqui's reagent) are also modifications (61). Some authors recommend spraying with a 0.5% solution of palladium chloride, with about 5 ug-sensitivity limit, which is selective for phenothiazines in general (58,59,61). Thioridazine reacts with this reagent with the formation of dark red spots. Similar reagents are ferric chloride, gold chloride and ceric sulfate (58,59); the first reagent (aqueous 2%) is a useful differentiating reagent for phenothiazines.

### 7.153 Thin-Layer Chromatography (TLC)

Solvent Systems: Owing to the drug reactivity in direct daylight and to oxygen, i.e., photo- and auto-oxidation, the chromatograms should be developed in dark (64) or at least that the development is not performed in direct light (61). According to the Kofoed et al (65), light alone is not the only cause of drug decomposition; the presence of oxygen is necessary. The use of inert gas, such as nitrogen, is recommended for drying of sample spots and the chromatogram to avoid autoxidation. Layers of silical gel G and FG or HF<sub>254</sub> are more frequently utilized. Thioridazine like other phenothiazines is relatively absorbed on silica gel; that is why many polar organic solvents are used for development sometimes with addition of ammonia or organic bases. Aqueous methanol 23:77, v/v; (62); methanol (66); methanol-n-butanol (6:1, v/v) (63); methanol-chloroform (1:1 v/v)(67); methanol-water-ammonium acetate (50:10:1.5, v/v/w) (65); n-propanol-1N ammonia (88:12, v/v)(62); acetone for silica prepared with 0.1N sodium hydroxide (67,68); acetone-ammonia (100:1, v/v)(67); acetone-benzene-ammonia (10:50:5, v/v/v)(69); methanol-methylal-ammonia (50:50:1, v/v/v) (66); and ethyl acetate-acetone; saturated with methylamine (57) are used for development of TL-chromatograms. The  $hR_f$ -values of thioridazine in some of these solvent systems are summarized in Table 8.

Table 8 : TLC  $hR_f$ -values of thioridazine and thioridazine sulfoxide (mesoridazine).†

<u>Adsorbent</u>	<u>Solvent System</u>	<u><math>hR_f</math> -value</u>		<u>Refer- ence.</u>
		<u>Thiori- dazine</u>	<u>Mesori- dazine</u>	
Silica gel G	70% Aqueous methanol	24	9	62
Silica gel	methanol	13	-	66
Silica gel	Chloroform-methanol (1:1)	32	-	67
Silica gel GF <sub>254</sub>	Ammonium acetate-water -methanol (3:20:100)	71	46*	65
Silica gel G	n-Propanol-1N ammonia (88:12)	39	13	62
Silica gel G	Benzene-acetone-ammo- nia (50:10:5).	45	17	70

† Detection as under 7.152

\*  $hR_f$ -value after oxidation with  $H_2O_2$  (65).7.154 Gas-liquid Chromatography (GLC)

Driscoll et al (71) studied the identification of phenothiazines by the GLC of their pyrolysis products. Variations in the amounts of many low-molecular-weight pyrolysis such as methane, ethylene, and propylene are sufficiently characteristic of the compounds to permit identification by their GLC-retention behaviour. GLC-separation and identification of thioridazine and thioridazine sulfoxide, i.e., mesoridazine, were studied by Kofoed et al (72), with FID and using stainless-steel or glass columns packed with 3% SE-30 on Gas Chrom Q. The sulfoxide gave only very broad peaks even by running at higher temperatures (73).

7.155 High-Performance Liquid Chromatography (HPLC)

Muusse and Huber (74) described an HPLC-system for elegant separation of

thioridazine, trace of impurities in bulk drug chemical and the main sulfone and sulfoxide metabolites of thioridazine on silica gel spherosil XOB using 2,2,4-Trimethyl pentane-2-aminopropane-acetonitrile-ethanol (96 : 0.96 : 2.4:0.48, v/v/v/v) as a mobile phase at a rate of 1.14 ml.mm<sup>-1</sup>. Detection was followed by UV (254 nm) and fluorescence (Ex: 365 nm & EM : 440 nm).

## 7.2 Quantitative

### 7.21 Determination in Bulk Materials

#### 7.211 Volumetry

##### i) Aqueous Titration

Thioridazine is alkylated by treatment with iodomethane in methanolic medium at 40-50°C for 30 min.; the resulting quaternary ammonium compound is titrated with ammonium thiocyanate (75).

##### ii) Non-aqueous Titration

The USP XXI (1) recommends the determination of thioridazine base or the hydrochloride salt by titrating the drug solutions in equal parts of glacial acetic acid and acetic anhydride against standard solution of 0.1 M acetous perchloric using potentiometry for end point's detection. The BP 1980 (2) describes another non-aqueous procedure by titrating the drug solution in acetone containing about 7% mercuric acetate solution against 0.1N acetous perchloric acid standard using saturated solution of methyl orange in acetone as indicator.

#### 7.212 Electrochemistry

##### i) Controlled-potential coulometry

Merkel and Discher (76) described an accurate controlled potential coulometric technique



for quantification of thioridazine by adopting two different supporting electrolytes but using platinum as the working electrode. Table 9 summarizes the overall analytical conditions and results.

Table 9 : Applications of Controlled Potential Coulometry for Quantification of Thioridazine using Platinum Working Electrode (77).

<u>Supporting electrolyte</u>	<u>Control potential,</u>	<u>Reaction</u>	<u>Sample weight,</u>	<u>Precision *</u>	<u>Reference</u>
	<u>V vs. SCE</u>		<u>mg</u>		
12-N $\text{H}_2\text{SO}_4$ in 30% ethanol (v/v).	+ 0.55	Oxidn	10-40	+0.17 mg	76
1-N $\text{H}_2\text{SO}_4$	+ 0.75	Oxidn	40	+0.4 mg	76

\* Precision given as standard deviation (+ mg) or coefficient of variation (%) as reported.

+ Date from more than one sample (3 replicates) or from different weight ranges were pooled to obtain the standard deviation (n-value is 1-2).

## ii) Voltammetry

Application of microliter vessels in voltammetric quantification of small sample volumes of thioridazine is described by Ebel et al (78). Oxidative voltammetry of thioridazine at 3-mm vitreous-carbon; i.e., stationary electrode (sample volume 80  $\mu\text{l}$ ), or a rotating-disc electrode (sample volume 1 ml) using dropping mercury electrode are applied for quantification of the drug.

## 7.213 Spectrophotometry

Ramappa and Basavaiah (79) described a colorimetric procedure for quantification of five phenothiazines including thioridazine hydrochloride in pure and in some dosage formulations.

## 7.214 Chromatography

### i) Combination of PC with Spectrophotometry

Densitometric evaluation of the spots of phenothiazines, including thioridazine, separated by PC can be made after the reaction with specific reagents, e.g. palladium chloride. Spectrophotometric determination after PC-separation is also recommended by using short-wave UV-light. Presentation of the drug on an ion exchanger, such as Amberlite IRC 50 and elution with citric acid-phosphate buffer pH4, then addition of 0.2 ml of 2% ghomma ghatti and 0.1 ml of iodoplatinate reagent followed by measuring the resulting color at 610 nm (61).

### ii) Combination of TLC with Spectrophotometry

Extraction of the separated spots of the drug on TLC with methanol or ethanol and spectrophotometric determination at 260 nm (61,80, 81). Bulenkov (82) recommends the extraction of the drug from thin layers by ether-isopropanol (28:2, v/v), followed by extraction into an acetate buffer and then colorimetric determination using palladium chloride reagent.

### iii) Combination of PC and TLC with other Chromatographic Methods

The separation of thioridazine with promazine, chlorpromazine, together with promethazine is achieved on thin layers, and the additional separation by GC-column is realized successfully.  $R_f$ -values calculated from elution data in centripetal TLC coincide with those for classical linear TLC. The combination of PC and TLC with other chromatographic methods seems to have good prospects for qualitative as well as quantitative analysis in pharmaceutical substances and related interest (61).

## 7.22 Determination in Pharmaceutical Formulations

### 7.221 Spectrometry

In addition to the colorimetric method described by Ramappa and Basavaiah (79) for assay of thioridazine in pure and dosage forms, there is another method for determination of thioridazine hydrochloride and sulforidazine in tablets by mixing their aqueous solution with diazotized 4-nitroaniline and conc. hydrochloric acid. The absorbances of the colored products are measured at 655 nm for thioridazine hydrochloride and at 510 nm for sulforidazine. The results of the colorimetric method were compared with those of official methods (83). The USP XXI (1) and the BP 1980 (2) recommend the spectrophotometric measurement at 266 nm for the thioridazine base in oral suspension using chloroform as the extracting solvent and the blank. For thioridazine hydrochloride in oral solutions, using ammoniacal chloroform, and in tablets, using chloroform, then the measurements are undertaken at 265 nm in both cases against the extracting solvent as the blank.

### 7.222 High-Performance Liquid Chromatography (HPLC)

Mehta (84) described a liquid chromatographic method for determination of thioridazine hydrochloride in syrups, injections or tablets. Solutions of the drug (in water or methanol) were analyzed on a column (25 cm x 5 mm) of Hypersil ODS (10  $\mu$ m) with aqueous 90% methanol containing 0.2% of ethanolamine as mobile phase and 265 nm detection; 0.2% cinchocaine hydrochloride was used as internal standard. Coefficient of variation was < 2% and results were in good agreement with those obtained by B.P. or B.P.C. methods. For identity, assay and content uniformity of thioridazine, a liquid chromatographic method is described by

Lovering et al (85). Solutions of the drug (in 1% HCl) were analyzed on a stainless-steel column (15 cm x 4.6 mm ) of Zorbax CN with 0.025-M sodium acetate buffer (pH 4.8)-acetotrile-methanol (4:7:9, v/v/v) as mobile phase (2.5 ml.min<sup>-1</sup>) and 254 nm detection; phenylpropanolamine was the internal standard. The coefficient of variation was < 2.5% for peak-area, < 1% for peak heights and < 2.2% (n=3) for analysis of tablets.

#### 7.223 Automated Flow-Injection

Koupparis and Barcuchova (86) described a single-manifold automated flow injection system with spectrophotometric detection for assay, content uniformity, and dissolution studies of thioridazine in pharmaceutical dosage formulations by using iron perchlorate. The drug solutions are fed at 2.4 ml min<sup>-1</sup> into an automated flow-injection analysis system with 2.75 mM ferric perchlorate in 10 M perchloric acid as oxidizing agent and water as carried solvent. The products are passed then to a spectrophotometer set at the  $\lambda$  max. of the drug. Measurement rate of 120 samples.hr<sup>-1</sup> can be attained with good precision. The method is evaluated by studying interference effects and recoveries and by the analysis of commercial formulations, the results of which are compared with those from the US Pharmacopoeial method. The technique is applied also in content uniformity testing and for monitoring the dissolution of solid dosage forms in 0.1M hydrochloric acid. The automated flow-injection analysis is applied also in case of some other phenothiazine antipsychotic drugs, namely chlorpromazine, promethazine, promazine, methotrimeprazine, thioproperazine, fluphenazine and trifluoperazine.

## 7.23 Determination in Tissues and Biological Fluids

### 7.231 Spectrophotometry and Spectrofluorometry

Thioridazine can be spectrophotometrically determined in urine after extraction (87,88). A non specific spectrofluorometric method for determination of thioridazine and some other phenothiazine drugs in plasma is described (89). Some of the non-conjugated metabolites of thioridazine, chlorpromazine, and trifluoroperazine can be detected and determined too. The drug is extracted with heptane, re-extracted into acetic acid and then oxidized with hydrogen peroxide to the corresponding fluorophores. The measurement is carried out against a blank of plasma; for thioridazine EX: 355 nm, EM: 430 nm, and analysis at 370-480 nm. This procedure distinguishes between the common phenothiazines but not between a drug and its metabolites in all cases. Pacha (90) described a similar spectrofluorimetric method for quantification of thioridazine and mesoridazine in plasma and, urine after treatment with 0.2-N  $\text{H}_2\text{SO}_4$  and 0.1%  $\text{KMnO}_4$ ; EX : 355 nm and EM : 440 nm.

### 7.232 Chromatography

#### i) Thin-Layer Chromatography (TLC)

Tewari (91) investigated the applicability of TLC using 18 systems for detection and determination of 22 different psychotropic drugs including thioridazine in toxicological screening.

#### ii) Gas-Liquid Chromatography (TLC)

Dinovo et al (92) described a GLC-procedure for the detection and determination of thioridazine and its major metabolites in plasma specimens. After alkalization of plasma, it is extracted with organic solvent mixture followed by several clean-up steps

and finally the GLC-analysis using chlorpromazine as internal standard. The separation can be achieved on 1.8 m x 2 mm i.d. glass column packed with 3% OV-17 on 100/120 mesh Chromosorb Q at 275°C, isothermally, using helium (100 ml.min<sup>-1</sup>). Debruyne et al (93) demonstrated the coupling of wall-coated open-tubular column with nitrogen-selective detector for routine GLC-determination of thioridazine in combination with some other drugs in serum using a glass column coated with SE-30 with nitrogen (40 ml.min<sup>-1</sup>) as the carrier gas and N-sensitive detector isothermally at 260°C with haloperidol as internal standard. Mårtensson et al (19) described a GLC-method for identification and quantification of thioridazine and its main non-conjugated metabolites. The separation is made on a 1 m x 3 mm i.d. glass column packed with 0.4% OV-225 on 80-100 mesh Gas-chrom Q at 240°C and for more polar metabolites at 270°C isothermally using nitrogen (85 ml min<sup>-1</sup>) as carrier gas and prochloroperazine dimaleate as internal standard. The structure elucidation of the isolated metabolites was undertaken by mass spectrophotometry; the base peak at m/e 98 is corresponding to C<sub>6</sub>H<sub>12</sub>N, while the base peak at m/e 84 is due to C<sub>5</sub>H<sub>10</sub>N the nor-thioridazine and the corresponding metabolites, i.e., demethylated compounds. Curry and Mould (94) recommended the use of a 1 m x 3 mm i.d. glass column packed with 3% OV-17 on Gas-Chrom Q at 260°C isothermally with nitrogen (50 ml.min<sup>-1</sup>) as carrier gas and prochloroperazine as internal standard. Axelsson and Mårtensson (35) have applied the last method for GLC-analysis of thioridazine and its main metabolites in biological specimens procedure for quantification of thioridazine and non-conjugated thioridazine metabolites in serum and urine of psychiatric patients is described by Mårtensson et al (19). The GLC-separation is undertaken on a 1 m x 3 mm i.d. glass column packed with 0.4% OV-225 on 80-100 mesh Gas-

Table 10 : GLC Analysis of Thioridazine and its Non-Conjugated Metabolites.

Compound	Specimen	Column; internal standard	Temp. (°C)	R <sub>t</sub> (min.)	Detection	Reference.
Thioridazine	Standard	2% SE-30 on chromosorb W(100-120 mesh); chlorpromazine.	220	12.0	FID	95
Thioridazine	Plasma	Open-tubular, % SE-30 coat; haloperidol.	260		N-FID	93
Thioridazine Non-conjugated metabolites	Plasma & urine	0.4% OV-225 on Gas-Chrom-Q (80-100 mesh); prochlorperazine	240 270	-	FID	19
Thioridazine & non-conjugated metabolites.	Plasma	3% OV-17 on Gas Chrom-Q; prochlorperazine.	260	-	FID	35,94
Thioridazine (T)	Plasma	3% OV-17 on Chromosorb Q (100-120 mesh); chlorpromazine	275	3.6 8.3 12.8 3.8 8.7 20.9 22.8	FID	92
T-2-sulfoxide (mesoridazine)						
T-5-sulfoxide						
Northioridazine (NT)						
NT-5-sulfoxide						
T-5-disulfoxide						
T-5-disulfone						

Chrom Q at 240°C using nitrogen (85 ml.min<sup>-1</sup>) as carried gas. Table 10 summarizes the different GLC conditions for separation and quantification of thioridazine and its metabolites.

iii) High-Performance Liquid Chromatography (HPLC):

Thioridazine, like other phenothiazines, has high degree of aromaticity and shows intense UV-absorption and fluorescence emission characteristics which have been used to advantage in the sensitive and specific determination of the drug by HPLC using UV and fluorescence detectors (73). The extensive literature on the thin-layer chromatographic separation of phenothiazines and their metabolites (96,97) can be adopted for developing HPLC systems using microparticulate silica gel or using reversed-phase chromatography with octa-decylsilane (ODS). Muusze and Huber (74) reported on a HPLC-procedure for quantification of thioridazine and its psychotropically active metabolites in blood. The separation and analysis are performed on a column containing 9 µm silica gel Spherosil XOB-03 using UV-detection at 254 nm. HPLC-resolution and quantification of thioridazine and mesoridazine in plasma is reported by McCutcheon (98). A column packed with µBondapak-C<sub>18</sub> is recommended for reversed-phase liquid chromatographic resolution of the drug and its 2-sulfoxide metabolite. Table 11 illustrates collectively the different HPLC-procedures recommended for identification and quantification of thioridazine with or without its main metabolites.

7.233 Radioreceptor Assay (RRA)

The clinical potency of thioridazine and other antipsychotic drugs is correlated to their potency in blocking dopamine receptors in the brain (108). Radioreceptor assay measures the total dopamine receptor-blocking



Table 11 : HPLC Determination of Thioridazine and Metabolites.

Compound	Sample	Column/temp.(°C)	Developing solvent or mobile phase	Retention time(min)	Detection	Sensitivity	Reference
Thioridazine (T) Northioridazine (NT) T-2-sulfoxide (sulfuridazine) T-2-sulfoxide (mesoridazine) T-5-sulfoxide NT-2-sulfoxide	Plasma	25 cm, 9- $\mu$ m silica gel Spherosil XDB at 25°C	2,2,4-Trimethylpentane (96%), 2-aminopropane (0.96%), acetonitrile (2.4%) and ethanol (0.48%) at 1.14 ml.min <sup>-1</sup>	1.2 1.6 2.2 2.7 4.0 5.7	UV (254 nm)  Fluorescence EX:365/Em:440 nm.	3-15 ng. $\mu$ l <sup>-1</sup>  3-15 ng. $\mu$ l <sup>-1</sup> (injected)	74
Thioridazine (T) T-2-sulfoxide	Plasma (whole)	25 cm, uBondapak-C <sub>18</sub> ambient.	Methanol (66%) and 1% Trifluoperazine acetic acid containing acid sodium salt (34%) at 2 ml.min <sup>-1</sup> .	8.5 3.9	UV (263 nm)	0.25-10 ng. $\mu$ l <sup>-1</sup>	98
Thioridazine (T) T-2-sulfoxide T-2-sulfone	Plasma	15 cm, LiChrosorb RP/8 ambient	Methanol (80%) and 7.6M sodium acetate (20%) containing 0.01% ammonium sulfate at 1 ml.min <sup>-1</sup>		Fluorescence after 200-W xenon mercury lamp irradiation.	= 0.5 ng (injected)	99
Thioridazi (T) T-2-sulfoxide T-2-sulfone	Plasma	On-line detector coupled to a suitable HPLC-column	Methanol (50%) and 0.01M acetate buffer pH 5(50%).		UV irradiation (28s) followed by fluoro- metric measurement.	=50-120 pg (injected) (0.01-4 ug. $\mu$ l <sup>-1</sup> )	100
Thioridazine (T) T-2-sulfoxide T-2-sulfone	Plasma	25 cm, 5- $\mu$ m spherisorb silica at 50°C. 7-methoxy- chlorpromazine in 1N NaOH.	Acetonitrile (85%) and methanol (15%) containing 0.03 butylamine butylamine at 2 ml min <sup>-1</sup> .		UV (254 nm)	0.05-2 ng. $\mu$ l <sup>-1</sup> (0.005 ng. $\mu$ l <sup>-1</sup> detectable).	101
Thioridazine (T) T-2-sulfoxide T-2-sulfone	Urine, plasma, bile, post- mortem tissues.	30 cm, 10- $\mu$ m Bondapak-C <sub>18</sub> / ambient.	Aqueous methanol (58%) containing 0.01M octylamine at 2 ml.min <sup>-1</sup> .		UV (265 nm)	4-45 ng. $\mu$ l <sup>-1</sup>	102
Thioridazine (T)	Plasma	25 cm LiChrosorb RP-8/ ambient	Aqueous methanol (70%) containing 0.1M LiH <sub>2</sub> O <sub>3</sub> , 0.05M tetraethylammonium perchlorate, 1mM KBr and 0.1M EDTA		Fluorescence EC:345/ Et:425 nm	2-20 ng(injected) =0.5 ng is detect- able in serum).	103

Table 11 contd...

Compound	Sample	Column/temp.(°C)	Developing solvent or mobile phase	Retention time(min)	Detection	Sensitivity	Reference
Thioridazine (T) Northloridazine (NT) T-2 or 5-sulfoxide T-2-sulfone	Plasma	5- $\mu$ m silica gel with precolumn filters.	2,2,4-Trimethylpentane (80%) methylene chloride (10%), and methanol (10%) containing 0.0367 methylamine, at 2.25ml.min <sup>-1</sup>		UV (254 nm)	20 ug. $\mu$ l <sup>-1</sup> (T-S-oxide) 10 ug. $\mu$ l <sup>-1</sup> (others).	104
T-5-sulfoxide diastereoisomeric pairs	Plasma & urine	5- $\mu$ m 10M silica/ambient after TLC separation on KAO-2 column with elution with ethyl acetate (85%) and propan-2-ol (15%).	Chlorobutane (92%), propan-2-ol (7.9), water (0.08%) and diethylamine (0.02%)		UV (279 nm) or MS at 70 eV	10-250 ug. $\mu$ l <sup>-1</sup> .	106
Thioridazine (T) T-2-sulfoxide T-2-sulfone T-5-sulfoxide (2 isomers)	Plasma	10- $\mu$ m spherical particle silica and precolumn solid-core/at 25°C. Trilupromazine.HCl.	1-chlorobutane (92%), 2-propanol (7.9%), water (0.08%), and diethylamine (0.016%), at 1.5 ml.min <sup>-1</sup>		Fluorescence EX = 300 EM: 500-600 nm.	0.20-0.4 ng. $\mu$ l <sup>-1</sup>	105
Thioridazine (T) Northloridazine (NT) T-2-sulfoxide T-5-sulfoxide	Plasma (Whole blood)	30 cm Cyano-bonded reversed phase column ambient. Chlorpromazine.	20 mM NaH <sub>2</sub> PO <sub>4</sub> containing 0.1M EDTA (70%) and Tetrahydrofuran (THF) (30%) at 1.5 ml min <sup>-1</sup>		amperometric (using glassy carbon electrode).	0.05-10 ug. $\mu$ l <sup>-1</sup>	107

activity in a sample and hence accounts for not only parent drug but active metabolites as well (109). RRA measures then total drug activity rather than the amount of the drug in a sample, this would allow clinicians to more accurately monitor patients receiving multiple neuroleptics and furthermore, such patients might then be included in one and the same drug level-response study. Tune et al (110) reported that RRA levels of thioridazine in serum were also higher than those of other neuroleptics. Mailman et al (111) have later questioned the analytical precision of RRA when used for thioridazine and its clinically active metabolites, using chlorpromazine or haloperidol as a standard. Radioreceptor assay was compared with HPLC for serum thioridazine and its major metabolites (37, 112).

#### 7.234 Radioimmunoassay (RIA)

Sensitive RIA-procedures have been developed for thioridazine and its metabolites (113, 114). The RIA-procedures have been compared with HPLC-methods. With regard to thioridazine, the RIA-procedures can measure as low as  $0.39 \text{ ng.ml}^{-1}$  thioridazine in plasma using a 200- $\mu\text{l}$  sample. Chakraborty et al (115) reported on the synthesis and properties of haptens for the development of radioimmunoassays for thioridazine, mesoridazine and sulforidazine.

#### ACKNOWLEDGEMENT

The authors would like to thank Mr. Altaf Hussain Naqvi for typing the manuscript.

REFERENCES

1. The United States Pharmacopoeia XXI, U.S. Pharmaceutical Convention, Inc., Rockville-Md, 1985, pp. 1054-1056.
2. The British Pharmacopoeia 1980, HM Stationary Office, London, 1980, pp. 453-454.
3. Clarke's Isolation and Identification of Drugs in Pharmaceuticals, Body Fluids, and Post-Mortem Materials, 2nd edn., A.C. Moffat (Editor), The Pharmaceutical Press, London, 1986, pp. 1019-1020.
4. The Merck Index, 10th edn., M. Windholz (Editor), Merck & Co., Inc., Rahway-NJ, 1983, No. 9202-p. 1341.
5. Remington's Pharmaceutical Sciences, 16th edn. A. Osol (Editor), Mack Publ. Co., Easton-Penn. (1980), p. 1036.
6. J.P. Tollenaere, H. Moereels and L.A. Raymackers (Editors): Atlas of the Three-Dimensional Structure of Drugs, 1st edn., Elsevier, Amsterdam-New York-Oxford, 1979, p. 120.
7. J.J.H. McDowell: Acta Cryst., B31, 2256 (1975).
8. Martindale's The Extrapharmacopoeia, 28th edn., J.E.F. Reynolds (Editor), The Pharmaceutical Press, London, 1982, p. 1559.
9. A.L. Green: J. Pharm. Pharmacol., 19, 10 (1967).
10. W.O. Foye (Editor): Principles of Medicinal Chemistry, 2nd edn., Lea & Febiger, Philadelphia-Penn., 1981, p. 908.
11. E. Pretsch, T. Clerc, J. Seibl and W. Simon (Editors), Tabellen zur Struktur-aufklärung organischer Verbindungen mit spektroskopischen Methoden, 2nd edn., Springer-Verlag, Heidelberg, 1981, C<sub>5</sub>-C<sub>265</sub>.
12. J., Renz., J.P., Bourquin, G., Gamboni and G., Schwarb; U.S. Patents: 3,239,514, assigned to Sandoz Ltd., Switzerland.

13. M. Sitting (Editor), Pharmaceutical Manufacturing Encyclopedia, Noyes Data Corporation, Park Ridge-NJ, 1979, p. 600.
14. J.P. Bourquin, G. Schwarb, G. Gamboni, R. Fischer, L. Ruesch, S. Guldemann, V. Theus, E. Schenker and J. Renz; Helv. Chim. Acta, 41, 1072 (1958).
15. Bentley and Driver's Textbook of Pharmaceutical Chemistry, L.M. Atherden (Editor), Oxford University Press, London, 1969, p. 674.
16. T.J. Mellinger; Am. J. Psychiatry, 121, 119 (1965).
17. DRUGDEX® , Micromedex 1977-1988, Rocky Mountain Drug Consultation Center, Denver General Hospital, Denver-Colorado, p. E04.
18. K. Zehnder, F. Kalberer, W. Kreis, and J. Rutschman; Biochem. Pharmacol., 11, 535 (1960).
19. E. Mårtensson, G. Nyberg and R. Axelsson; Curr. Ther. Res., 18, 687 (1975).
20. H. Freeman, H. Rivera, M. Oktem and N. Oktem; Curr. Ther. Res., 11, 263 (1969).
21. A. Zabrowski; Exp. Med. Psychiat., 25, 297 (1971).
22. H. Haase, M. Bergener and H. Hasselmeijer; Med. Welt, 18, 542 (1967).
23. N. Petrilowitsch; Int. Pharmacopsychiat., 1, 230 (1968).
24. G. Buyze, P.F.C. Egberts, R.G. Muusze and A. Poslavy; Psychiat. Neurol. Neurochir., 76, 229 (1973).
25. M. Niedzwiecki, R.B. Mailman, and L.X. Cubeddu; J. Clin. Exp. Ther., 228, 636 (1984).
26. R. Axelsson and E. Mårtensson, Curr. Ther. Res., 28, 463 (1980).

27. M.L. Rao, W.A. Brown and R. Wagner; Ther. Drug Monit., 10, 184 (1988).
28. C.D. Kilts, R.B. Mailman, E. Hudson, and G.R. Breese; Fed. Proc., 40, 238 (1981).
29. E. Widerlov, J.-E. Haggstrom, C.D. Kilts, U. Andersson, G.R. Breese, and R.B. Mailman; Acta Psychiatr. Scand., 66, 294 (1982).
30. R.C. Young and R.W. Nyswander; Res. Commun. Psychol. Psychiatr. Behav., 9, 345 (1984).
31. S.L. Thompson; Acta Pharm. Tox., 26, 298 (1968).
32. R.C. Baselt (Editor), Disposition of Toxic Drugs and Chemicals in Man, Part 1, 2nd edn., Biomedical Publications, Davis-CA, 1978, p. 112.
33. R.C. Baselt, J.A. Wright and E.M. Gross; J. Anal. Tox., 2, 41 (1978).
34. G. Nyberg, R. Axelsson and E. Mårtensson; Eur. J. Clin. Pharmacol., 13, 34 (1978).
35. R. Axelsson and E. Mårtensson; Curr. Ther. Res., 19, 242 (1976).
36. G.S. Avery (Editor), Drug Treatment, Principles and Practice of Clinical Pharmacology and Therapeutics, ADIS-Press, Sydney, 1976, p. 894.
37. G. Nyberg, C. Svensson, U. Olofsson, R. Axelsson and E. Mårtensson; Ther. Drug Monit., 9, 426 (1987).
38. J.M. Silver, S.C. Yudofsky, M. Kogan, and B.L. Katz; Am. J. Psychiatry, 143, 1290 (1986).
39. R.M. Greendyke and A. Gulya; J. Clin. Psychiatry, 49, 105 (1988).
40. Drug Information 88-AHFS, G.K. McEnovy, American Society of Hospital Pharmacists, Inc., Bethesda-MD, 1988, p. 1174.

41. R. Axelsson and E. Mårtensson; Curr. Ther. Res., 21, 561 (1977).
42. Drug Information 88-AHFS, G.K. McEvoy (Editor), American Society of Hospital Pharmacists, Inc., Bethesda-MD, 1988, p. 1158-1164.
43. H.H.A. Standish-Barry and M.A. Shelly; Lancet, 1, 771 (1983).
44. E.W. Martin (Editor), Drug Interaction Index 1978/9, Lipponcott Co., Phil., 1978, p. 209.
45. R.J. Robbins, P.A. Kern, and T.L. Thompson; Am. J. Med., 76, 921 (1984).
46. A.W. Marcoux; Hosp. Pharm., 21, 889 (1986).
47. C.D. Sands, J.D. Robinson, R.B. Salem, R.B. Stewart, and C. Muniz; Drug Intell. Clin. Pharm., 21, 267 (1987).
48. M.A. Moustafa, S.A. Babhair and H.I. Kouta; Int. J. Pharm., 36, 185 (1987).
49. R.E. Gosselin, R.P. Smith, and H.C. Hodge (Editors), Clinical Toxicology of Commercial Products, 5th edn., Williams & Wilkins, Baltimore-London, 1984, p. II-1425.
50. T.J. Haley, A.M. Flesher, K. Raymonds, N. Konesu, and P.A. Williams; Toxicol. Appl. Pharmacol., 1, 377 (1959).
51. R.H. May, P. Selymes, R.D. Weekley, and A.M. Potts; J. Nerv. Ment. Dis., 130, 230 (1960).
52. E.I. Goldenthal; Toxicol. Appl. Pharmacol., 18, 185 (1971).
53. J.S. Gillis; Curr. Ther. Res., 21, 224 (1977).
54. MEPPO, The Middle East Drug Compendium, 17th edn, HCP-Healthcare Publications, Nicosia-Cyprus, 1988, p. 510.
55. Clarke's Isolation and Identification of Drugs in Pharmaceuticals, Body Fluids, and Post-Mortem Materials, 2nd edn., A.C. Moffat (Editor), The Pharmaceutical Press, London, 1986, p. 258., p. 134.

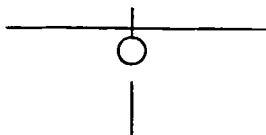
56. V. Jokl and J. Dolejšova: Cesk. Farm.; 26, 283-286 (1977).
57. K. Macek, J. Vecerkova, and J. Stanislakova; Pharmazie, 20, 605 (1965).
58. J. Vecerkova, M. Sulcova, and K. Kac!; J. Chromatogr., 7, 527 (1962).
59. J. Vecerkova, M. Sulcova, and K. Kac!; Pharmazie, 17, 22 (1962).
60. H. Street; Pharm. Acta Toxicol., 19, 312 (1962).
61. K. Macek (Editor), Pharmaceutical Applications of Thin-Layer and Paper Chromatography, Elsevier Publ. Co., London, 1972, p. 98.
62. T.J. Mellinger and C.E. Keelr; J. Pharm. Sci., 51, 1169 (1962).
63. J. Cochin and J.W. Daly; J. Pharm. Exptl. Ther., 139, 160 (1963).
64. W. Rusiecki and M. Henneberg; Acta Polon. Pharm., 21, 23 (1964).
65. J. Kofoed, C. Fabierkiewicz, and G.H.W. Lugas; J. Chromatogr., 23, 410 (1966).
66. W. Awe and W. Schulze; Pharm. Ztg., 107, 1333 (1962).
67. A. Noirfalise and M.H. Grosjean; J. Chromatogr., 16, 236 (1964).
68. I. Zingales; J. Chromatogr., 31, 405 (1967).
69. F. Eiden and H.D. Stachel; Deut. Apotheker-Ztg., 103, 121 (1963).
70. W. Paulus, W. Hotch, and R. Keymer; Arzneim.-Forsch., 13, 609 (1963).
71. J.L. Driscoll, H.F. Martin, and B.J. Gudzinowicz; J. Gas Chromatogr., 2, 109 (1969); through ref. 73.



72. J. Kofoed, C. Fabiarkiewicz, and G.H. Lucas; J. Chromatogr., 23, 410 (1966).
73. J.A.F. De Silva, GLC and HPLC Determination of Therapeutic Agents, K.Tsuji (Editor), part 2, Marcel Dekker, Inc., New York-Basel, 1978, pp. 593-600.
74. R.G. Muusze and J.F.K. Huber; J. Chromatogr. Sci., 12, 779 (1974).
75. D. Zivanov-Stakic and L. Djceric; Arch. Pharm., 27, 223 (1977).
76. F.H. Merkel and C.A. Discher; Anal. Chem., 36, 1639 (1964).
77. R.E. Schirmer (Editor), Modern Methods of Pharmaceutical Analysis, Vol. 2, CRC-Press, Inc., Boca Raton-Fl., 1982, p. 207.
78. S. Ebel, E.-J. Placke, and P. Surmann; Fresenius Z. Anal. Chem., 321, 660 (1985); through Anal. Abstr., 48, 4E3 (1986).
79. P.G. Ramappa and K. Basavaiah; Indian J. Pharm. Sci., 47, 125 (1985).
80. W. Rusiecki and M. Henneberg; Acta Pol. Pharm., 23, 117 (1966) through Anal. Abstr., 14, 4251 (1967).
81. J. Sugita and Y. Tsujino; J. Chromatogr., 21, 321 (1966).
82. T.I. Bulenkov; Med. Prom. SSSR, 17, 26 (1963) through C.A., 60, 5280e (1964).
83. S.R. El-Shabouri; Talanta, 32, 999 (1985).
84. A.C. Mehta; Analyst (London), 106, 1119 (1981).
85. E.G. Lovering, N.Beaulieu, R.C. Lawrence and R.W. Sears; J. Assoc. Off. Anal. Chem., 68, 168 (1985).
86. M.A. Koupparis and A. Baruchova; Analyst (London), 111, 313 (1986).

87. I.S. Forrest, F.M. Forrest and S.L. Kanter, J.E. Sperco, and M.B. Wechsler; Am. J. Psychiat., 121, 1049 (1965); through Biol. Abstr., 47, 7316 (1966).
88. I.S. Forrest, F.M. Forrest and S.K. Kanter; Clin. Chem., 12, 379 (1966).
89. J.B. Ragland, V.J. Kinross-Wright, and R.S. Ragland; Anal. Biochem., 12, 60 (1965).
90. W.L. Pacha; Experientia, 25, 103 (1969).
91. S.N. Tewari; Zentralbl. Pharm. Pharmakother. Laboratoriumsdiagn., 116, 1123 (1977).
92. E.C. Dinovo, L.A. Gottschalk, B.R. Nandi, and P.G. Geddes; Anal. Chem., 65, 667 (1976).
93. D. Debruyne, M.A. Moulin, R. Camsonne, and M.C. Bigot; J. Pharm. Sci., 69, 835 (1980); through Anal. Abstr., 40, 3D49 (1981).
94. S.H. Curry and G.P. Mould; J. Pharm. Pharmacol., 21, 674 (1969).
95. A.P. DeLeenheer; J. Chromatogr., 77, 339 (1973).
96. C. Korczak-Fabierkiewicz and G.J. Cimbura; J. Chromatogr., 53, 413 (1970).
97. P.N. Kaul, M.W. Carway, and M.L. Clark, Nature; 226, 372 (1970).
98. J.R. McCutcheon; J. Anal. Toxicol., 3, 105 (1979).
99. A.H.M. Scholtan, P.L.M. Welling, U.A.T. Brinkman and R.W. Frei; J. Chromatogr., 199, 239 (1980).
100. A.H.M. Scholten, U.A.T. Brinkman and R.W. Frei; Anal. Chim. Acta., 114, 137 (1980).
101. T. Skinner, R. Gochnauer, and M. Linnoila; Acta Pharmacol. Toxicol., 48, 223 (1981).
102. W.J. Allender; J. Chromatogr. Sci., 23, 541 (1985).

103. W.T. Kok, W.H. Voogt, U.A.T. Brinkman, and R.W. Frei; J. Chromatogr., 354, 249 (1986).
104. C.D. Kitts, K.S. Patrick, G.R. Breese, and R.B. Mailman; J. Chromatogr. (Biomed. Appl.), 231, 377 (1982).
105. C.E. Wells, E.C. Juenge, and W.B. Furman; J. Pharm. Sci., 72, 622 (1983).
106. P.W. Hale and A. Poklis; J. Chromatogr. (Biomed. Appl.), 336, 452 (1984).
107. A.L. Stoll, R.J. Baldessarini, B.M. Cohen, and S.P. Finklestein; J. Chromatogr., 307, 457 (1984).
108. R.I. Greese, D.R. burt, and S.H. Snyder; Science, 192, 481 (1976).
109. S.G. Dahl; Clin. Pharmacokin., 11, 36 (1986).
110. L.E. Tune, I. Greese, J.R. DePaulo, P.R. Slavney and S.H. Snyder; J. Nerv. Ment. Dis., 169, 60 (1981); through reference 107.
111. R.B. Mailman, J.P. Pierce, K.M. Crofton, J. Petitto, and D.L. DeHaven; Biol. Psychiatry, 19, 833 (1984); through reference 107.
112. M.L. Rao, W.A. Brown, and R. Wagner; Ther. Drug Monit., 10, 184 (1988).
113. B.S. Chakraborty, I. Rosenthahler, E.M. Hawes, and K.K. Midha, Ther. Drug Monit., 9, 227 (1987).
114. B.S. Chakraborty, E.M. Hawes and K.K. Midha, Ther. Drug Monit., 9, 464 (1987).
115. B.S. Chakraborty, M.S. Sardessa, T.J. Jaworski, and K.K. Midha; Pharm. Res., 4, 207 (1987).



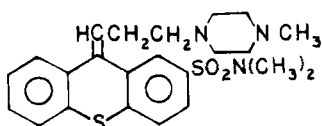
THIOTHIXENE

Dorothy K. Wyatt, Lee T. Grady

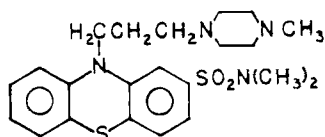
1. Introduction
2. Description
  - 2.1 Name, Formula, Molecular Weight
  - 2.2 Appearance, Color, Odor
3. Synthesis
4. Physical Properties
  - 4.1 Infrared Spectrum
  - 4.2 Nuclear Magnetic Resonance Spectra
  - 4.3 Ultraviolet Spectrum
  - 4.4 Mass Spectrum
  - 4.5 Melting Range
  - 4.6 Solubility
  - 4.7 Moisture Content
  - 4.8 Isomerism
  - 4.9 X-Ray Crystallography
  - 4.10 Fluorescence Spectroscopy
5. Methods of Analysis
  - 5.1 Elemental Analysis
  - 5.2 Color Tests
  - 5.3 Spectrophotometric Analysis
  - 5.4 Fluorescence Analysis
  - 5.5 Paper Chromatography
  - 5.6 Thin-Layer Chromatography
  - 5.7 Gas-Liquid Chromatography
  - 5.8 High Performance Liquid Chromatography
6. Degradation-Stability
7. Pharmacokinetics
  - 7.1 Absorption
  - 7.2 Distribution
  - 7.3 Metabolism
  - 7.4 Excretion
8. Determination in Biological Fluids
  - 8.1 Plasma
  - 8.2 Blood
  - 8.3 Urine
  - 8.4 Other
9. Determination in Pharmaceuticals
10. References

## 1. Introduction

Thiothixene was synthesized in 1967 [1] as the third in a series of thioxanthene derivatives. The thioxanthene structure is similar to the phenothiazine moiety and thiothixene is the thioxanthene analog of thioperazine [1,2]. The piperazine moiety common to both these drugs is believed to increase lipid solubility [3] whereas the sulfonamide function potentiates tranquilizer activity [4]. The thioxanthenes are more stable in solution and less sensitive to photooxidation than the phenothiazines [5]. Thiothixene has been reported as being approximately equipotent [6,7,8,9,10] and also less potent [2,4] than its corresponding analog. Increased clinical efficacy with less side effects than trifluoperazine has also been reported for thiothixene at recommended maximum thiothixene dose [11].



thiothixene



thioperazine

Thiothixene is an antipsychotic drug which is used mainly in the treatment of both acute and chronic schizophrenia. It has also been effective in anxious depressed patients [12] and is especially active in disorders of perception, thought content and processes, insight and judgment [13]. Improvement in hallucinatory behavior or irritability, social competence and personal neatness has been shown [9]. Symptoms such as mannerisms, suspiciousness, tension, withdrawal, hostility, and disorientation also seem to be considerably decreased [9].

Thiothixene disrupts conditional avoidance behavior in rats at low doses (3.2 mg/kg ip) [7,9,14] and in monkeys [14]. It blocks apomorphine induced emesis in dogs [7,14] at less than 5 µg/kg iv. Thiothixene blocks hyperactivity [7,9], stereotyped symptoms and mortality rates caused by amphetamines in mice and rats [7]. It exhibits only very weak anticholinergic, antihistaminic, hypotensive, hypothermic [8,14], and sedative properties in animals [14]. It is very weak in disrupting escape behavior in rats, in potentiating hexobarbital or ethanol induced loss of righting reflex, and in eliciting flaccidity in rats [7]. Thiothixene

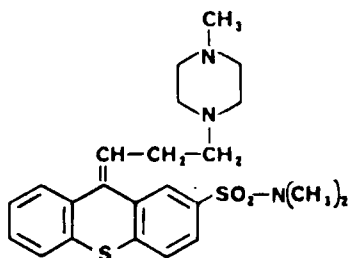
also induces catalepsy in rats and both catalepsy and tremors in dogs and monkeys [7,15].

Thiothixene is believed to act at four anatomical sites: the reticular activating system of the midbrain, the amygdala and the hippocampus of the limbic system, the hypothalamus, and the globus pallidus and corpus striatum [2]. The exact biochemical mechanism of action is unknown. However, thiothixene is believed to act by decreasing central dopaminergic transmission either by directly inhibiting the dopamine receptor or by inhibiting the post-synaptic action of a dopamine sensitive adenylate cyclase [2].

Neuroleptic drugs have been shown to be active inhibitors of the dopamine sensitive adenylate cyclase [16]. In addition, structural similarity to dopamine has been postulated for the neuroleptics [13,17]. Receptor blockage by a dopamine-like drug might then lead to a compensatory increase in activity of the dopaminergic cells by a neuronal feedback mechanism [16,17]. Thiothixene has been shown to increase synthesis and turnover of dopamine resulting in an elevation of dopamine metabolites in the brain and cerebrospinal fluid [18]. This increase in synthesis is in approximate proportion to clinical potency [19]. Increased brain concentration of dihydroxyphenylacetic acid (DOPAC) and of homovanillic acid (HVA) has been observed in the striatum of rodents [18]. Increased homovanillic acid concentration has been observed in the cerebrospinal fluid of psychotic patients after treatment [20,21]. The elevation of prolactin concentration in serum [18,21] and in cerebrospinal fluid [21] has also been observed. This is believed to be mediated by the blockage of hypothalamic and/or pituitary dopamine receptors by neuroleptics; an alpha-adrenergic blocking effect also occurs [21,24]. However, the exact mechanism of action of neuroleptics and specifically thiothixene and their action in schizophrenia awaits further definition.

## 2. Description

### 2.1 Name, Formula, Molecular Weight, Registry Number



Thiothixene

$C_{23}H_{29}N_3O_2S_2$

molecular weight: 443.62

CAS Registry No.: 5591-45-7

(Z-) 3313-26-6

## 2.2 Synonyms

N,N-Dimethyl-9-[3-(4-methyl-1-piperazinyl)propylidene]thioxanthene-2-sulfonamide [22,23,24,25,27]

9-H-Thioxanthene-2-sulfonamide, N,N-dimethyl-9-[3-(4-methyl-1-piperazinylpropylidene)-, (Z-)] [22,25]

cis-9-[3-(4-Methyl-1-piperazinyl)propylidene]-2-(dimethylsulfonamide)thioxanthene [23]

cis-2-Dimethylsulfamoyl-9-[3-(4-methyl-piperazin-1-yl)propylidene] thioxanthene [6]

Tiotixene, Navane, Orbinamon, Navaron (obsolete) [23,25]

## 2.3 Appearance, Color, Odor, and Taste

Thiothixene is a white to tan almost odorless crystalline powder [26].

## 3. Synthesis

See following pages.

## 4. Physical Properties

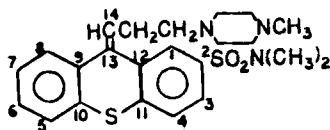
### 4.1 Infrared Spectroscopy

Principal bands of a thiothixene potassium bromide dispersion are given in Table I [28,30]. A typical spectrum of a potassium bromide dispersion is presented in Figure 1. The spectrum can also be determined in a 1 in 20 chloroform dispersion of the drug in 0.1 mm cells [22].

Table I  
Infrared Characteristics of Thiothixene

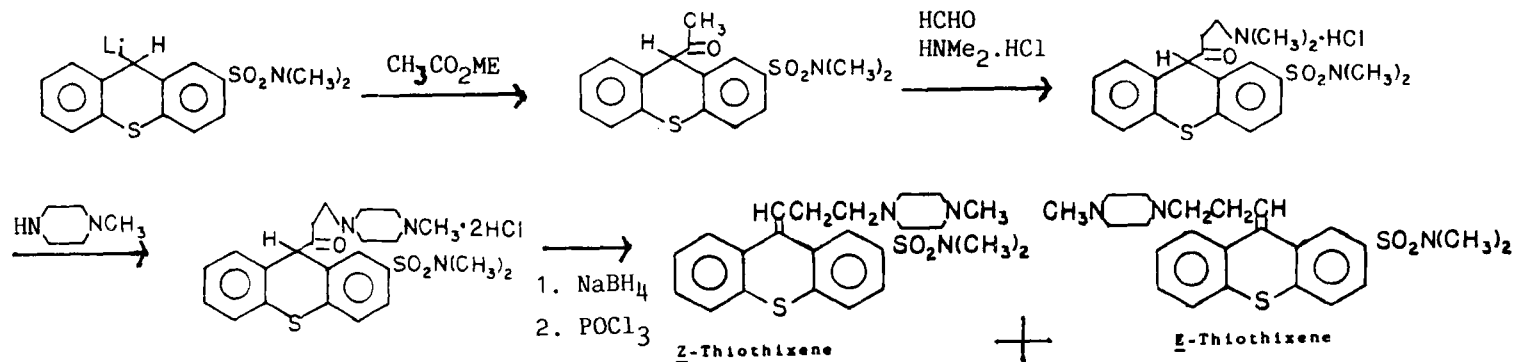
$\mu$	assignment
6.1	C=C
7.5	SO <sub>2</sub>
8.7	SO <sub>2</sub>
12.	vinyl CH

### 4.2 Nuclear Magnetic Resonance Spectroscopy

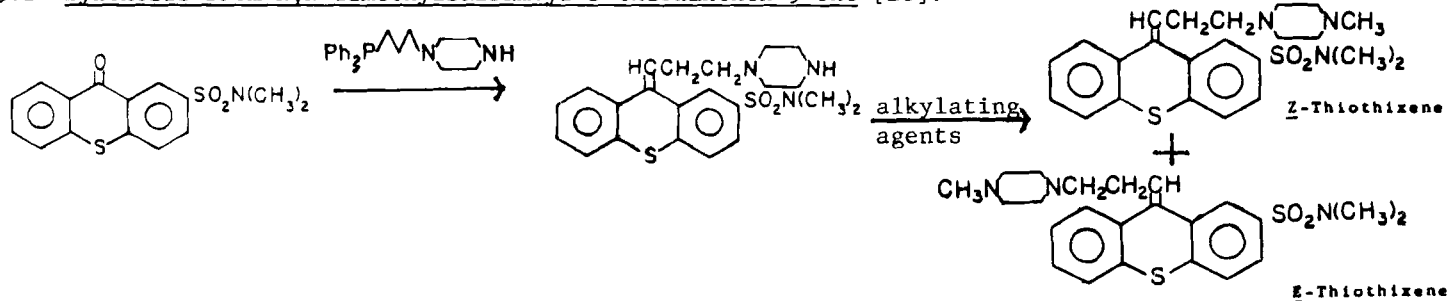




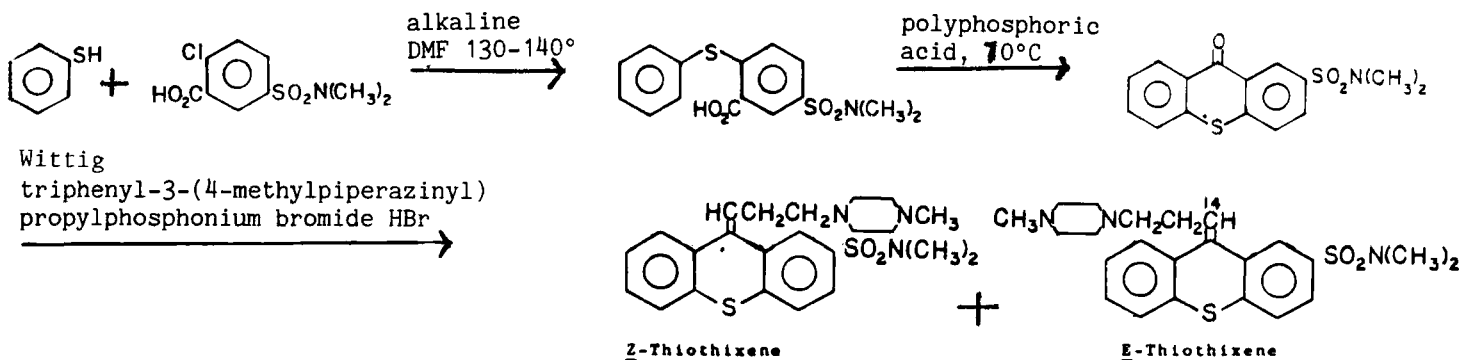
### 3.1 Synthesis from 9-lithio-N,N-dimethylthioxanthene-2-sulfonamide [28].



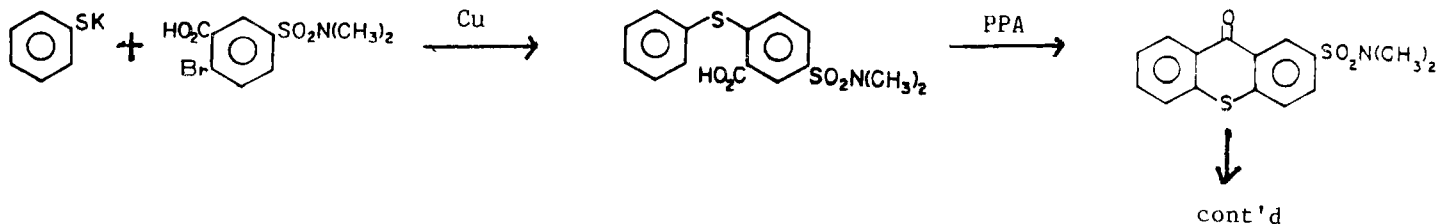
### 3.2 Synthesis from N,N-dimethylsulfamoyl-2-thioxanthen-9-one [28].



### 3.3 Synthesis from thiophenol and 2-chloro-5-dimethylsulfamoylbenzoic acid [29].



### 3.4 Synthesis from potassium thiophenoxide and 2-bromo-5-dimethylsulfamoylbenzoic acid [28].





#### 4.2.1 Proton Spectra

The proton spectra are presented in Figures 2 and 3 and spectral assignments in Table II [31]. Spectra were obtained using a Bruker AM-400 and solutions of 25 mg/mL in deuteriochloroform in 5 mm o.d. glass NMR tubes. Proton NMR was accomplished using sweep widths corresponding to approximately 11 ppm and acquisition times of approximately 2 seconds. Proton spectra are also reported using a Varian T-60A NMR operating at 60 MHz. A solution of drug in deuteriochloroform-1% TMS was used in the analysis. A sweep width of 500 Hz and an acquisition time of 250 seconds was used [30]. Proton NMR assignments were also obtained using a Varian A-60 spectrometer [28,32]. Concentrations and instrument parameters were unspecified. Data are reported in Table II.

Table II

#### Proton NMR Spectral Assignments for Thiothixene

Chemical Shift ppm	Multiplicity of Proton	Characteristic	Reference
7.81	singlet	1-H	31
7.60	doublet	3-H	
7.57	doublet	4-H	
7.48	doublet	8-H	
7.35	doublet	5-H	
7.31	triplet	6-H	
7.24	triplet	7-H	
6.01	triplet	14-H	
2.73	singlet	(CH <sub>3</sub> )SO <sub>2</sub> -	
2.62	quartet	-CH <sub>2</sub> CH=	
2-54	triplet	-CH <sub>2</sub> N-	
2.52, 2.45	multiplet	piperazine	
2.27	singlet	CH <sub>3</sub> N-	
2.28	singlet	CH <sub>3</sub> N-	28
2.72	singlet	(CH <sub>3</sub> )SO <sub>2</sub> -	
6.03	multiplet	14-H	
7.2-7.7	multiplet	aromatic CH	
7.86	multiplet	1-H	
7.3-7.8		aromatic CH	32
6		14-H	
9.3-8.4		aromatic CH	32*

\*Spectra obtained in sulfuric acid solution.

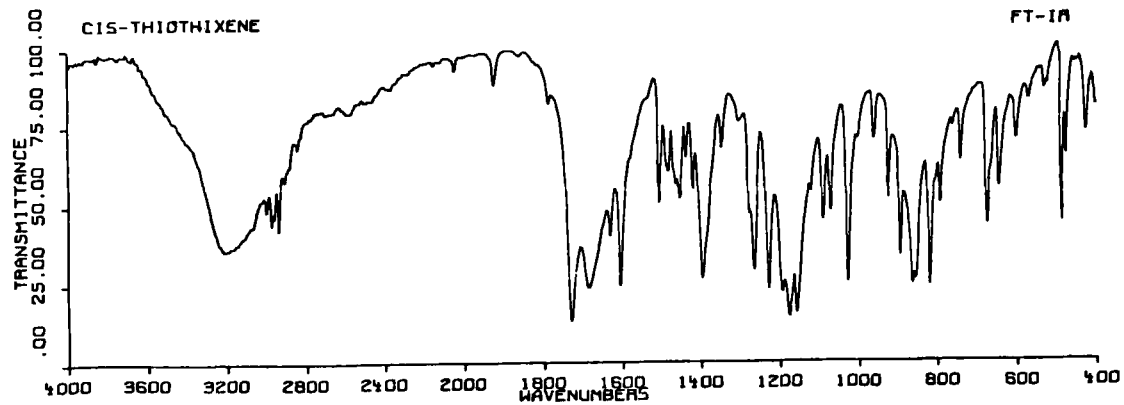


Fig. 1. Infrared spectrum of thiothixene: potassium bromide dispersion.

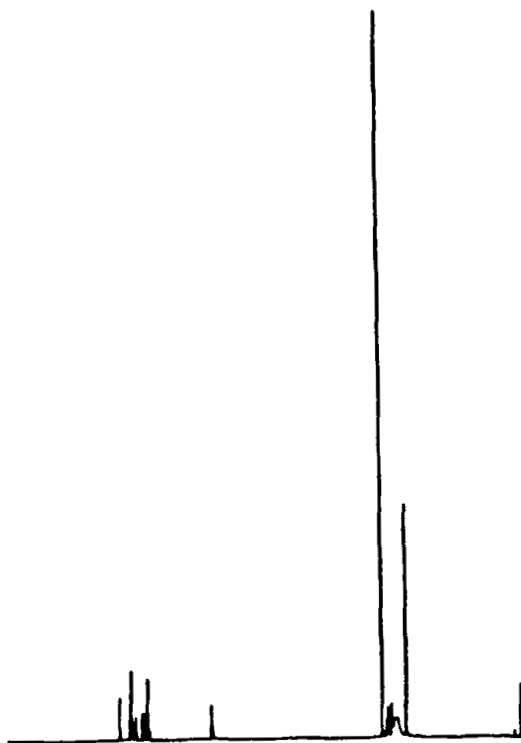


Fig. 2. Thiothixene proton NMR.

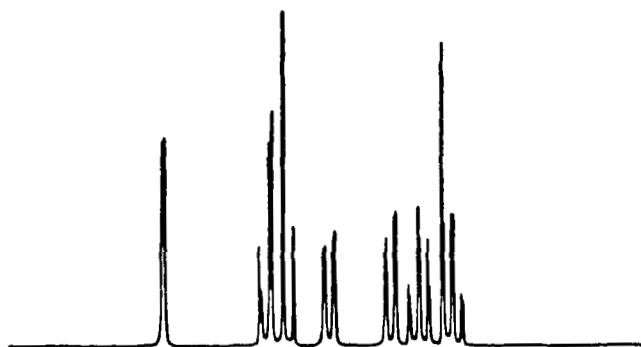


Fig. 3. Thiothixene proton NMR: downfield signals.

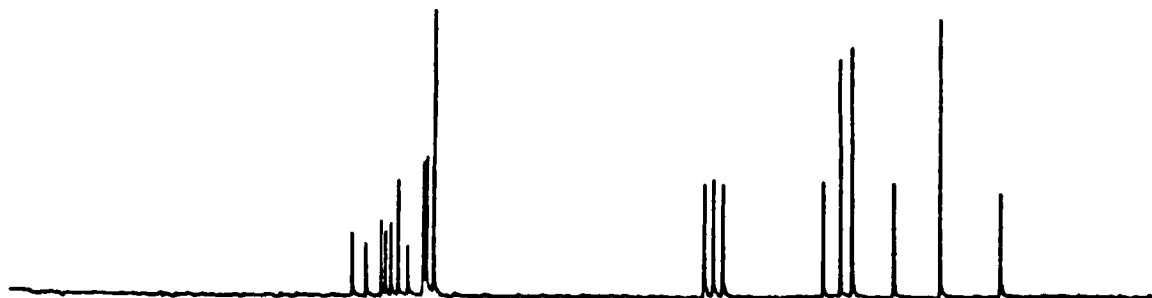


Fig. 4. Thiothixene  $\text{C}^{13}$  NMR.



Fig. 5. Thiothixene  $\text{C}^{13}$  NMR: downfield signals.

#### 4.2.2 Carbon<sup>13</sup> NMR Spectra

The carbon<sup>13</sup> NMR spectra are presented in Figures 4 and 5 and spectral assignments in Table III [31]. Spectra were obtained using a Varian FT-80A NMR and solutions of 25 mg/mL in deuteriochloroform in 5 mm o.d. glass NMR tubes. Carbon<sup>13</sup> NMR was accomplished using sweep widths corresponding to approximately 200 ppm and acquisition times of approximately 2 seconds.

Table III

Carbon<sup>13</sup> NMR Spectral Assignments for Thiothixene

<u>Chemical Shift (ppm)</u>	<u>Carbon Number</u>
140.1	11
137.7	12
135.1	13
134.2	9
133.4	2
132.1	14
130.4	10
127.7	1
127.5	6
127.2	7
127.0	4
125.9	3
125.8	8
125.8	5

#### 4.3 Ultraviolet Spectroscopy

The ultraviolet absorption spectrum of thiothixene is given in Figure 6. Absorbance and wavelength maxima are given in Table IV.

#### 4.4 Mass Spectroscopy


The electron impact mass spectrum is presented in Figure 7. The mass spectrum was recorded using a LKB-9000 gas chromatograph-mass spectrometer. An ionizing voltage of 29 ev was used and the ion source was maintained at 290 C. A GC column containing 1% Pentasil TM-350 on Gas Chrom Q (80-100 mesh) at 285 C was used. Fragments at 443 and 113 at M<sup>+</sup> and CH<sub>2</sub>=N+NCH<sub>3</sub>, respectively [35]. The electron impact mass spectrum was also acquired using a Hewlett-Packard 5985B gas chromatograph-mass spectrometer. An electron energy of




Table IV

Ultraviolet Wavelengths and Absorptivity of Thiiothixene

<u>Solvent</u>	<u>Wavelength (nm)</u>	<u>Absorptivity</u>	<u>Reference</u>
methanol	230		22
	307		
methanol	228	4.6	23
	260	4.3	
	310	3.9	
methanol	228	4.6	28
	260	4.2	
	310	3.9	
0.1 N sulfuric acid	228		25
	257		
	308		
	228	850*	33
	308	140*	
	257		
concentrated sulfuric acid	286		32
	387		
	489		
0.2 N sulfuric acid	227		30
	307		
strongly basic	309		30
0.1 M hydrochloric acid	229		34
alkaline pH	310	120*	33

\*E<sup>1%</sup><sub>1 cm</sub>

70 ev was used. The samples were injected onto a 3% OV-1 packed column and introduced via a glass jet separator into the ms source which was maintained at 200 C [30].

The chemical ionization mass spectrum is shown in Figure 8. The mass spectrum was obtained using a Finnigan model 3200E gas chromatograph-mass spectrometer. A 500 microamp emission current, electron multiplier at 1800 volts, electron energy at 100 ev, and a preamplifier range of 10<sup>-9</sup> amps/volt were used. A GC column consisting of 3% SP-2250DB on Supelcoport (100-120 Mesh) at 250 C was used. Fragments at 443 and 113 are M<sup>+</sup> and CH<sub>2</sub>=N+NCH<sub>3</sub>, respectively. A quasi-molecular (M + 1)<sup>+</sup> at m/e 444 and a molecular adduct ion at m/e 472 (M + 29)<sup>+</sup> were also observed. Trans-thiiothixene exhibited a fragment at 447 which was not observed for the cis-isomer [36].

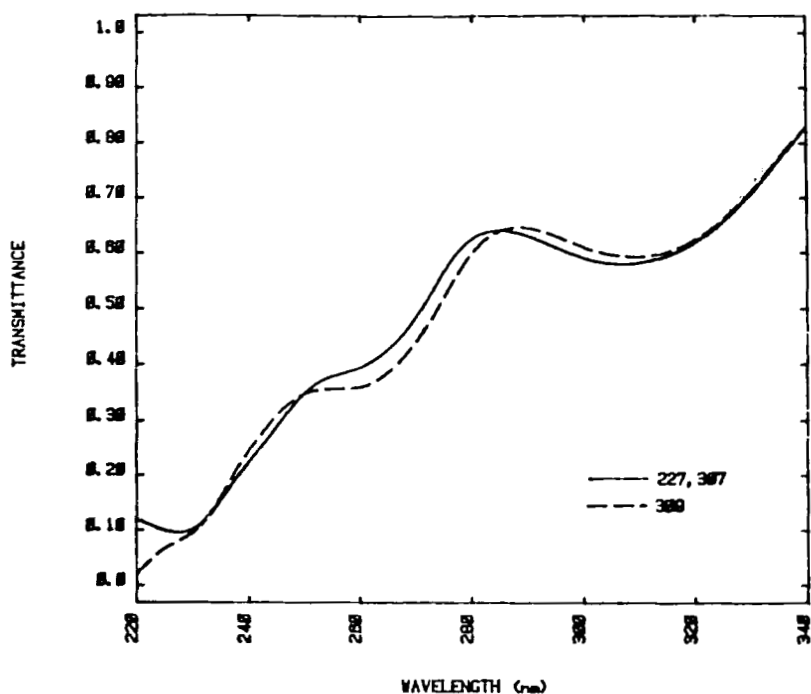


Fig. 6. Ultraviolet spectrum of thiothixene.  
 — 0.2 N sulfuric acid  
 - - - strongly basic

#### 4.5 Melting Range

Melting ranges are reported in Table V.

Table V

Melting Ranges for Thiothixene

<u>Temperature (C)</u>	<u>Conditions</u>	<u>Reference</u>
147-152	Class I	22
147.5-149		22,28
147-152		24,26

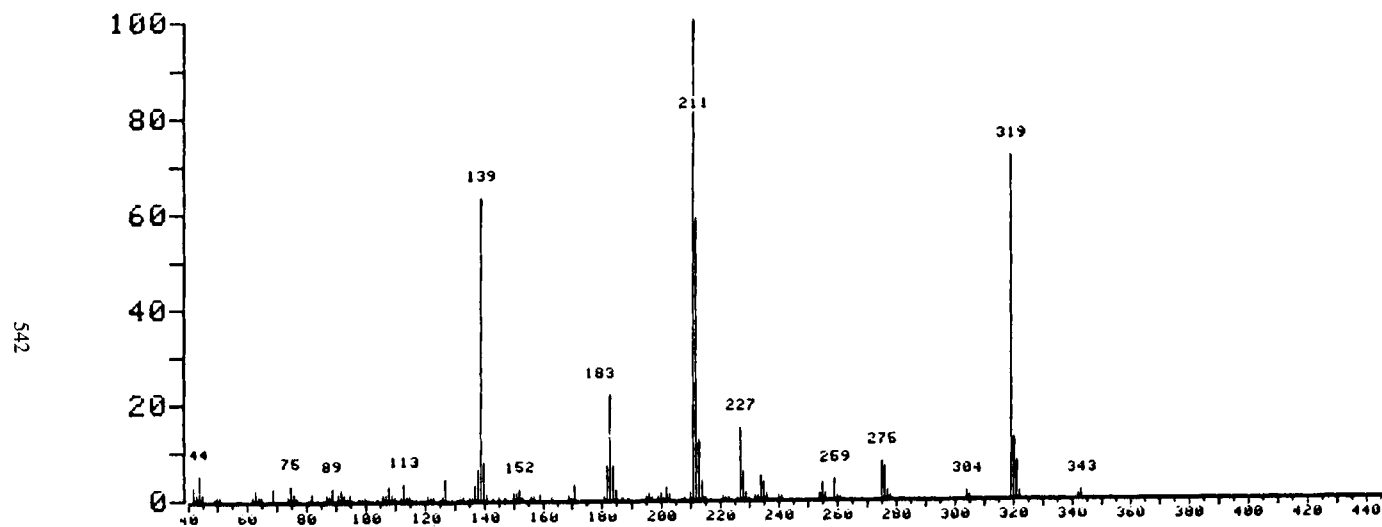


Fig. 7. Electron Impact Mass Spectrum.

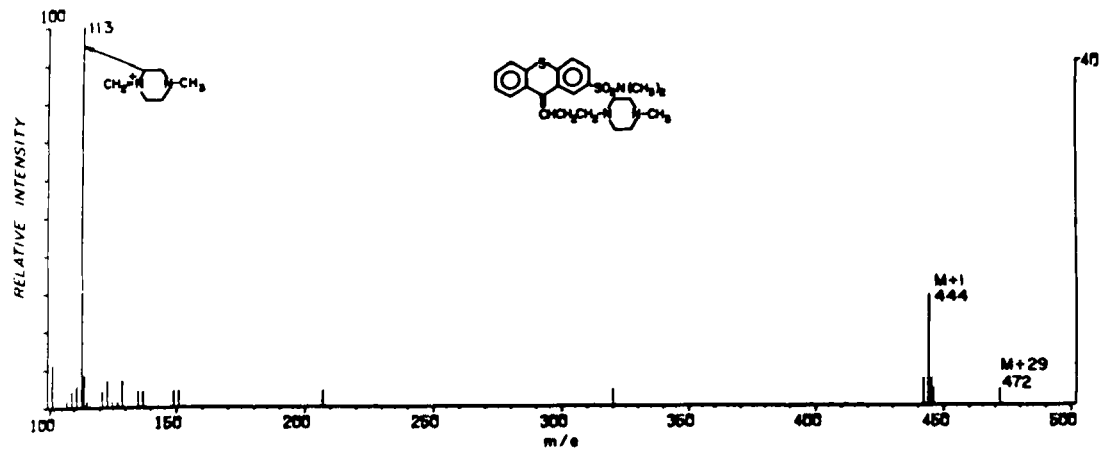


Fig. 8. Chemical Ionization Mass Spectrum of Thiothixene.

#### 4.6 Solubility

The approximate solubilities are reported in Table VI.

Table VI  
Solubilities of Thiothixene

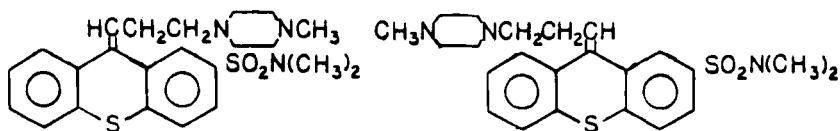
<u>Solvent</u>	<u>Solubility</u>	<u>Reference</u>
water	practically insoluble	24,26
alcohol	soluble	24
alcohol	slightly soluble	26
chloroform	very soluble	24,26
acetone	slightly soluble	24,26
methanol	slightly soluble	24
carbon tetrachloride	slightly soluble	26

#### 4.7 Moisture Content

Thiothixene has been dried at 100°C for three hours in vacuo [22].

#### 4.8 Cis-trans Isomerism

Thiothixene exists in the two isomeric forms shown below.



Cis- (Z-) Thiothixene

Trans- (E-) Thiothixene

Cis- and trans- thiothixene both are formed during synthesis. Either isomer is converted readily into an equilibrium mixture consisting of 37% cis-thiothixene [8,18,21, 22]. Conversion has been accomplished by irradiation of the cis or trans isomer which has been stored under nitrogen [37]. Either isomer can also be dissolved in 2 N aqueous hydrochloric acid and heated for four hours to produce the

equilibrium mixture [28]. In vivo conversion of isomers has also been reported [36]. However, only the cis isomer is biologically active [36,37,38,39,40].

#### 4.9 Crystal Structure

The molecular structure is given in Figure 9. The crystal structure [40] was determined as  $a = 10.13$ ,  $b = 8.77$ ,  $c = 19.99$  Å,  $B = 139.8$ , space group  $P2_1$ ,  $Z = 2$ ,  $D_m = 1.294$ ,  $D_c = 1.285$  for  $2(C_{23}H_{29}N_3O_2S_2)$  monoclinic crystals. This information was collected for Okl-5kl and h01-h31 and the intensities of 2131 reflections were measured visually from equi-inclination Weissenberg photographs [40]. The inter-atomic distances were reported as C-C aromatic 1.414, C-C (single bond) 1.545, C-C (vinyl) 1.514, C-C (vinyl-phenyl) 1.456, C-C 1.401, C-N 1.465, C-S 1.740, S-O 1.453 Å [40]. Least squares planes through the aromatic rings were calculated and the maximum separation of an atom from the surface of the plane was 0.01 Å. The two planes intersect at an angle of  $141.5^\circ$  [40].

The torsion angles are given in Figure 10. The geometrical characteristics derived from crystallographic data were also reported [41] as given in Table VII. A Rotran program was used for the analysis. No instruments were specified.

Table VII

#### Geometrical Characteristics of Thiothixene

<u>Distances (Å)</u>		<u>Angle I AA'</u>	<u>Z-Coordinates</u>	
AA'	4.9	142°	ZA <sub>N</sub>	1.2
AN	7.4		ZA <sub>NLP</sub>	0.8
ANLP	7.9		ZA' <sub>N</sub>	2.5
A'N	6.1		ZA' <sub>NLP</sub>	2.1
A'NLP	6.8			

#### 4.10 Fluorescence Spectroscopy

The excitation and fluorescence maxima for thiothixene are 335 nm and 385 nm, respectively as measured using a Baird Atomic SF 100E spectrofluorometer fitted with a 150-w xenon source. Excitation and emission slits were fixed to provide a spectral bandpass of 6 nm [42]. The fluorescence maxima has also been reported as 380 nm using a Bearn

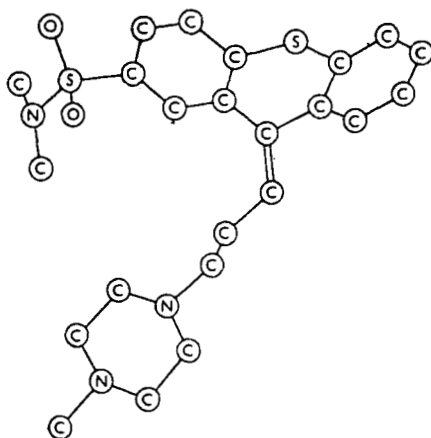


Fig. 9. Crystal Structure of thiothixene

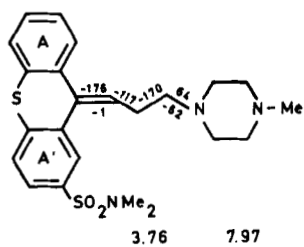


Fig. 10. Torsion angles of thiothixene

spectrofluorometer (slit width unspecified) fitted with a xenon source [43]. Thiothixene exhibits no phosphorescence [42].

## 5. Methods of Analysis

### 5.1 Elemental Analysis [23]

<u>atom</u>	<u>percent</u>
carbon	62.27
hydrogen	6.59
nitrogen	9.47
oxygen	7.21
sulfur	14.46

### 5.2 Color Tests

<u>Agent</u>	<u>Color</u>	<u>Sensitivity</u>	<u>Reference</u>
sulfuric acid-formaldehyde	red	0.1 $\mu$ g	25
ammonium molybdate	red	0.1 $\mu$ g	25
ammonium vanadate	red	0.25 $\mu$ g	25
Vitali's test	red-faint green	0.1 $\mu$ g	25
sulfuric acid	orange		32

### 5.3 Spectrophotometric Analysis

The official NF XIV (1975) content uniformity procedure for thiothixene capsules is spectrophotometric [44]. One opened capsule and shell is transferred to a 50-mL volumetric flask and 30 mL of dilute methanolic hydrochloric acid (1 in 120) is added. The solution is shaken for 10 minutes. Dilute methanolic hydrochloric acid is added to volume and mixed. A portion of this solution is centrifuged. This portion is sequentially diluted to produce a solution containing approximately 20  $\mu$ g/mL of thiothixene. Concomitant analysis of this solution and a solution of reference standard in the same medium at a concentration of about 20  $\mu$ g/mL in 1 cm cells at a maximum wavelength of about 307 nm allows determination of thiothixene content.

The official USP XXI [22] dissolution test for thiothixene capsules is a colorimetric determination using methyl orange. Forty mL of the test preparation, a dissolution medium consisting of 2.0 g sodium chloride and 7 mL of hydrochloric acid in water per liter, and standard preparation containing about 1  $\mu$ g/mL of thiothixene reference standard in dissolution medium are transferred to individual



separators containing 8.0 mL of phosphate buffer solution and 10 mL of methyl orange solution, and 50 mL of chloroform. The separators are shaken for 3 minutes and 40 mL of the chloroform layer is transferred to an additional separator containing 8 mL of 1 in 120 dilute hydrochloric acid. The separator is shaken for 1 minute and the layers allowed to separate. The absorbance of the aqueous layers is determined in 1 cm cells at the wavelength of maximum absorbance at about 508 nm.

#### 5.4 Fluorescence Analysis

After oxidation with 0.1% potassium permanganate and reduction of the excess potassium permanganate with 0.1% hydrogen peroxide, the fluorescence of the oxidized drug was determined using an Aminco Bowman spectrofluorometer using 310 nm activating and 440 nm fluorescent wavelengths. The sensitivity was estimated at less than 1 nm of pure drug [45].

#### 5.5 Paper Chromatography

Thiothixene was analyzed on Whatman #1 paper buffered by dipping in a 5% solution of sodium dihydrogen citrate. The developing solvent consisted of 4.8 g citric acid in a mixture of 130 mL of water and 870 mL of n-butanol. An  $R_f$  of 0.25 was obtained. Visualization was accomplished using ultraviolet light (blue fluorescence) and potassium iodoplatinate (purple). Bromocresol green produced a weak reaction [25].

Analysis was also accomplished using Whatman 31 filter paper buffered by dipping in pH 2.1 aminoacetic acid buffer. The developing solvent was prepared by mixing 100 mL of benzene, 50 mL chloroform, and ethylene glycol to saturate the immiscible layers. The upper layer is used in the analysis. Just prior to applying the samples to the paper, the paper is washed with 60% methanol in ethylene glycol. Visualization was accomplished using ultraviolet light (blue fluorescence) and potassium iodoplatinate (purple). Trans-thiothixene is separated in this procedure [22].

Thiothixene can also be analyzed using the NF XIV [44] procedure. Whatman #4 filter paper and a mobile phase consisting of the upper layer of a solution of 200 mL of ethyl acetate, 20 mL of butyl alcohol, and 20 mL of water which has been shaken and allowed to separate. The chamber is allowed to equilibrate with mobile phase for 16 hours prior to use in the analysis. The thiothixene is identified

using ultraviolet light at an  $R_f$  of approximately 0.4.

### 5.6 Thin Layer Chromatography

Thiothixene was analyzed on silica gel G plates using methanol-stronger ammonia water (100:1.5) as the mobile phase. The plates were visualized with acidified potassium iodoplatinate. An  $R_f$  of 0.45 was obtained for thiothixene [25]. Thiothixene was also analyzed on silica gel G plates that had been prewashed with dimethyl formamide-hydrochloric acid (9:1). Thiothixene had an  $R_f$  of 0.30 after visualization under ultraviolet light. Thioxanthone is separated in this system [43].

Thiothixene was analyzed using silica gel GF plates and diethyl ether-diethylamine (9:1), acetone-n-heptane-diethylamine (6:4:1), and benzene-dimethylformamide-diethylamine (8:1:1) systems.  $R_f$  values of 0.28, 0.40, and 0.66 were obtained respectively after visualization under ultraviolet light and by spraying with concentrated sulfuric acid. Separation from N-demethylthiothixene was obtained in all three systems. Thiothixene sulfoxide was separated completely in the first two systems and had an  $R_f$  of 0.60 vs. 0.66 for thiothixene in the third system [45]. Thiothixene can also be analyzed on silica gel GF plates using a system of ethyl acetate-diethylamine-water (90:15:5) and detection by autoradiography [29].

Thin layer chromatography using methanol-12 N aqueous ammonia (100:1.5) on silica gel plates was accomplished [46] using Fast Blue Salt B and potassium iodoplatinate solution of Folin-Ciocalteu as visualization agents. An  $R_f$  of 0.84 was obtained relative to phenmetrazine. Thin layer chromatography was also accomplished using chloroform-methanol (9:1) on silica gel G plates. The drug was visualized using ultraviolet light (254 nm), methanolic iodine solution, modified Ludy-Tenger reagent, or methanolic iodine solution and copper chloride [34].

Thiothixene was analyzed using benzene-methanol-25% aqueous ammonia (40:20:5) and silica gel G plates. The  $R_f$  of thiothixene was 0.4. Detection was accomplished using Dragendorff's reagent [47]. Thiothixene was also analyzed on silica gel GF plates using ethyl acetate-methanol-aqueous ammonia (85:10:5), methanol-aqueous ammonia (100:1.5), and isopropyl ether-ethanol (8:2). Drug was identified using acidified iodoplatinate [33]. Using these systems,  $R_f$ s of 0.57, 0.43, and 0.09, respectively, were obtained. Analysis [65] was also realized using silica gel GF plates and

n-butanol-acetic acid-water (4:1:1) and methanol-25% aqueous ammonia (100:1.5) systems. After visualization using ultraviolet light (254 nm),  $R_f$ s of 0.19 and 0.92 were obtained.

Thiothixene was also analyzed on silica gel G plates [48] using chloroform-methanol-25% aqueous ammonia (70:10:5) [ $R_f$  = 0.78], benzene-acetone-25% aqueous ammonia (40:40:5) [ $R_f$  = 0.34], chloroform-methanol-benzene-25% aqueous ammonia (90:5:5:1) [ $R_f$  = 0.57], ethyl octane-heptane (9:1) [ $R_f$  = 0.42], and chloroform-heptane (8:2) [ $R_f$  = 0.33] systems. Visualization was accomplished using Dragendorff's reagent. Silica gel G plates and a system consisting of ethyl acetate-methanol-diethylamine (65:35:5) has also been used [44]. Iodoplatinate was used as a detection agent.

### 5.7 Gas Liquid Chromatography

Thiothixene was analyzed using a 1% Pentasil TM-350 on Gas Chrom Q (80-100 mesh) column (1.5 mm x 30 cm) at 285 C using a LKB-9000 gas chromatograph-mass spectrometer. The retention time of thiothixene was one minute. The injector temperature was 310°C. Conditions for the mass spectrum were described in section 4.4 [35]. A GC/MS procedure was also developed using a 3% SP-2250-DB on 80-100 mesh Supelcoport column (2 mm x 40 cm) at 250°C. Thiothixene elutes at 4 minutes. Trans-thiothixene elutes at a relative retention time of 1.8 compared to the cis-thiothixene [36].

The drug was also analyzed [49] using a Hewlett-Packard 5880A gas chromatograph and nitrogen phosphorus detector. The injection port and detector were maintained at 250°C and 300°C, respectively. The drug was injected in butyl acetate. A temperature program was run [190°C-220°C (5°C/min), 220°C-280°C (15°C/min), 280°C-300°C (8°C/min), 300°C (5 minutes)]. Retention time relative to cyclizine was 4.6. Additional analyses were accomplished using a 3.8% SE-30 GC column at a temperature of 220°C. Retention time relative to dibenzepin was 5.4 minutes [46].

### 5.8 High Pressure Liquid Chromatography

Thiothixene was analyzed on silica gel of controlled surface porosity bonded to a solid spherical core, 30  $\mu$ m to 50  $\mu$ m in diameter using a mobile phase prepared by mixing 1400 mL of ethanolamine-methanol (0.5 mL of 3780 mL) with 200 mL of water. The column was maintained at ambient temperature. An ultraviolet detector at 254 nm is used for quantitation [22]. Thiothixene was analyzed using a Corasil II column and a mobile phase consisting of 0.37 mL of

ethanolamine and 400 mL of water diluted to 2.8 mL with methanol. Ultraviolet detection at 254 nm was used. N-(1-naphthyl)ethylenediamine dihydrochloride was used as the internal standard [50].

Separation of isomers and analysis of thiothixene was accomplished on Spherisorb (5  $\mu$ m) column (25 cm x 4.0 mm) using a mobile phase consisting of ethyl acetate-methanol-3% w/v ammonia (85:15:1). Ultraviolet detection at 200 nm was used for the determination. Ambient temperature and a flow rate of 1 mL/min was used [38]. Thiothixene isomers were also separated [51] using a radial compression module (RCM-100) and Radial-Pak Nitrile CN (10  $\mu$ m, 10 cm x 8 mm) cartridge. Mobile phases consisting of methanol-acetonitrile-0.03 M sodium dihydrogen phosphate-triethylamine (400:50:50:1) which was adjusted to pH 7.45 with phosphoric acid (a) or methanol-acetonitrile-0.03 M sodium dihydrogen phosphate-triethylamine (650:100:250:1) at pH 3.7 (b) were used in the analysis. Mesoridazine was used as the internal standard. Ultraviolet detection at 254 nm was used. Cis- and trans-thiothixene retention times were 7 and 8 minutes using mobile phase (a). Analysis [52] was also conducted using a Spherisorb cyanopropyl column (5  $\mu$ m, 150 x 4.6 mm) and a mobile phase consisting of 0.01 M potassium dihydrogen phosphate (pH 7.0)-acetonitrile-methanol (400:480:120) at a flow rate of 2 mL/min. Ultraviolet detection at 229 nm was used. Trans-thiothixene had a retention time of 1.24 relative to the cis-isomer. Thiopropazine was used as the internal standard.

Thiothixene isomers, precursors, and degradation products were separated using a 5  $\mu$ m SiAl (16% w/w alumina in silica column (10 cm x 4.6 mm) at 225 nm using a mobile phase of 10 mM  $\text{LiH}_2\text{PO}_4$ , pH 5.5, in 60% acetonitrile at a flow rate of 3 mL/min. Analyses on bulk drug and pharmaceutical preparations and dissolution testing can be accomplished using this method [53].

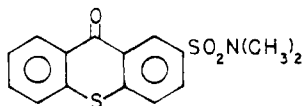
Thiothixene was also analyzed using a Varian Micropak CN (10  $\mu$ ) column and a mobile phase of 10% 0.005 M ammonium acetate in methanol at 2.5 mL/min flow. Ultraviolet detection at 254 nm was used. A retention time of 8.2 minutes was obtained [54]. Using a similar mobile phase of 10% 0.005 M ammonium acetate in methanol or acetonitrile and a Waters radial compression column at a flow rate of 5 mL/min and either ultraviolet detection at 254 nm or electrochemical detection at 10 nA and a voltage of +0.9 v (sensitivity, 0.01 ng/mL) analysis of thiothixene was also realized [55,56].

Using a mobile phase consisting of methanol-2 M ammonium hydroxide-1 M ammonium nitrate (27:2:1) with 50 mg of sodium sulfite added to each liter, thiothixene was analyzed on silica, mercaptopropyl modified silica, and n-propyl sulfonic acid modified silica which had been slurry packed into 25 cm x 5 mm I.D. stainless steel columns. Thiothixene  $k'$  values were 0.4, 0.9, and 0.1, respectively [57].

Thiothixene was analyzed on silica RP18 and Micropak MCH 10 (30 cm x 4 mm) columns. The mobile phase consisted of acetonitrile-perchlorate solution (5 mM perchloric acid and 15 mM sodium perchlorate) at ratios of 3:7, 4:6, and 6:4. Flow rate was 1.7 mL/min. Ultraviolet detection at 230 nm was used in the determination [58].

## 6. Degradation-Stability

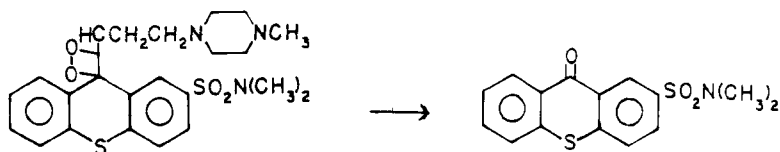
The major decomposition product of thiothixene is 2-(N,N-dimethyl-sulfonamido)-9-thioxanthone [43,59].



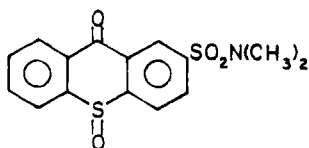
Photooxidation occurs in millimolar neutral or dilute acid solution resulting in the formation of the fluorescent [43], nontoxic [43], light yellow precipitate of the compound shown above [59]. Storage in the light in methanol solution at elevated temperature for 1, 12, and 18 months at 37 C and 50 C yields increasing decomposition with time [43]. Overall decomposition was less than 0.6%. Decomposition was also observed after storage in amber bottles in chloroform solution at 50 C [43, no impurity was observed initially]. It is indicated that formation of this compound can be prevented by the absence of direct irradiation or by degassing the solution under nitrogen [59].

Photooxidation is attributable to either addition of a singlet oxygen to the olefin resulting in an intermediate adduct such as dioxetane (shown below) which then collapses to the thioxanthone impurity or by the formation of a charge

transfer complex with oxygen which then rearranges to a hydroperoxide [59].



Chemical oxidation of thiothixene with potassium permanganate yields thioxanthone sulfoxide [58]. This reaction is utilized in a reported fluorescence assay [44].



## 7. Pharmacokinetics

### 7.1 Absorption

Thiothixene is rapidly absorbed following oral administration [29]. Patients are given between 6 and 60 mgs daily in divided doses [24]. A 20 to 60 mg dose is usually given [35]. The therapeutically effective plasma concentration in humans has been reported as 10.0 to 22.5 ng/mL [35]. The  $ED_{50}$  (intraperitoneal dosing) was 0.3 mg/kg (antiamphetamine) for mice and 1.0 to 3.2 mg/kg (antiavoidance) for rats [28].  $LD_{50}$  values of 100 mg/kg [28] and 55 mg/kg [29] for mice and rats (intraperitoneal dosing) were obtained, respectively.

An absorption half-life of approximately 0.5 hours was obtained based on human plasma studies [35]. An early disappearance half-life of 3.5 hours and a late disappearance half-life of 34 hours were observed [35]. Peak plasma levels were obtained 1 to 3 hours after administration of the daily final dose [35]. In rat studies, an early half-life of 3 days and a later half-life of approximately 4.5 days were observed for the liver. Plasma levels declined more rapidly [29].

Reabsorption from the intestine is postulated to occur (enterohepatic circulation) [29,35] since little to no thiothixene is excreted unchanged in rats [29] and a resurgence of thiothixene concentration in the plasma of some subjects has been observed [35].

Probable conversion in vivo of cis-thiothixene to the biologically inactive trans-isomer has been reported following human plasma studies and quantitation of the trans-thiothixene content [36]. Concentration of the trans-isomer varied between 0 and 36% of the total plasma thiothixene measured. All patients had been given thiothixene containing 0.5 to 1.0% trans-thiothixene. Mediation by an unknown isomerase at low gastric pH conditions prior to absorption has been postulated [36].

## 7.2 Distribution

Thiothixene is widely and rapidly distributed in the tissues of rats [29]. Distribution after a single dose (8 mg/kg of thiothixene-26<sub>s</sub>) is given in Table VIII for tissues examined 4 hours after dosing. At 4 hours after intraperitoneal dosing, all tissues examined had higher levels of radioactivity.

Table VIII

Tissue Levels ( $\mu$ g Thiothixene Equivalent/gm) in Rats

	<u>Intraperitoneal</u>		<u>Oral</u>	
	4 hour	24 hour	4 hour	24 hour
heart	1.12,1.50	0.14,0.09	0.21,0.13	0.14,0.05
lung	4.75,2.05	0.70,0.80	0.85,0.90	0.09,0.10
liver	11.43,7.91	4.42,5.91	4.96,7.17	6.60,5.71
kidney	1.50,0.46	0.61,0.38	0.47,0.68	0.42,0.19
stomach	23.50,10.69	0.23,0.46	9.12,14.35	0.04,0.05
skin	1.65,1.31	0.23,0.32	0.04,0.05	0.28 $\pm$ 0.01
muscle	1.20,1.25	0.09,0.14	0.04,0.09	0.03,0.02
brain	0.23,0.09	0.02,0.04	0.04,0.02	0.01,0.01

Distribution after eight doses is given in Table IX. Equilibrium was reached after the fifth dose. In both these cases, the liver showed the highest concentration of thiothixene one or more days after dosing. The liver was the only organ with appreciable amounts of thiothixene remaining after three days.

Table IXTissue Levels ( $\mu$ g Thiothixene Equivalent/gm) in Rats

	<u>Days After Last Dose</u>		
	1	2	3
heart	1.07	0.61	0.81
lung	2.08	1.67	1.87
liver	21.35	15.62	12.32
kidney	3.58	1.77	2.96
stomach	3.37	3.05	2.84
muscle	0.98	0.23	0.66
brain	0.06	0.03	0.21

High levels of thiothixene were reported in the stomach after single intraperitoneal or oral dosing. This was postulated to be due to the basicity of thiothixene and its metabolites coupled with the acidity of the gastric mucosa. Since intraperitoneal dosing produced a similar response, the phenomenon is not associated with the retention of drug in the stomach following oral administration.

Levels of thiothixene in the brain were the lowest of all body tissues examined after single or multiple dosing. Distribution in rat brain tissue one hour after a single intraperitoneal dose (10 mg/kg) of labeled thiothixene is reported in Table X. Relatively higher levels of thiothixene were observed in the gray matter compared to the white. Only unchanged drug was detected in the brain one hour after administration. Additional testing was not conducted at later intervals.

Table XDistribution of Radioactivity within the Rat Brain

	$\mu$ g Thiothixene Equivalent/gm
cerebral hemispheres	0.72
olfactory bulb	0.66
cerebellum	0.54
paraflocculus	0.43
thalamus	0.60
medulla	0.59
corpus callosum	0.73

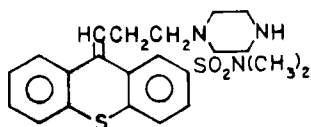


Levels of thiothixene in the eye remained stationary for several days and then declined. The rate of decline was slower than that of the liver. Thiothixene accumulates in the pigmented choroid which is 8% of the total eye weight so that concentration in the choroid may actually be quite high relative to the liver concentration. Thiothixene has some affinity for melanin and, hence, pigmented areas of the body such as the skin of rats or the choroid in the eye would have a higher concentration of thiothixene.

### 7.3 Metabolism

The liver is the major site of thiothixene metabolism. Thiothixene is rapidly metabolized with little of the drug excreted unchanged [29]. Some adverse effects on hepatic excretory function have been reported in rats [5]. Bile samples obtained within 0.5 hours of drug administration contain the same range of metabolites as later urine and bile samples.

The major metabolite of thiothixene in man [35,50], and rats and dogs [29] has been identified as N-dimethylthiothixene. The thioxanthene moiety is believed to be excreted unchanged [35]. With the exception of possible N-methyl fragments, thiothixene does not appear to be incorporated into normal tissue constituents [44].



N-Demethylthiothixene

### 7.4 Excretion

Thiothixene is excreted mainly in the bile of both rats and dogs studied although dogs did excrete a greater fraction in the urine [29]. A 5:2 ratio of biliary to urinary excretion was obtained for dogs [29]. Little to no thiothixene is excreted unchanged with no discernible changes in the pattern of metabolites during the course of excretion [29].

## 8. Determination in Biological Fluids

### 8.1 Plasma

A GC/EIMS procedure using a 1% Pentasil TM-350 column has been developed for the determination of thiothixene in plasma (sections 4.4, 5.7) [35]. The plasma samples were prepared by adding 80 ng of the internal standard, trideuterothiothixene, to 4 mL of plasma. The solution was made alkaline by adding 4 drops of 2 N sodium hydroxide and then extracted first with diethyl ether and then with 5 mL of ether-hexane (3:1). The combined organic extracts were dried over sodium sulfate and the solvent evaporated under dry nitrogen. The residue was dissolved in 0.1 mL of 0.01 M methanolic acetic acid and 0.05 mL aliquots were analyzed by GC/MS using m/e 113 (thiothixene) and m/e 116 (trideuterothiothixene) fragments for quantitation [35]. Sensitivity was determined to be less than 1 ng/mL of plasma. The ratio of the peaks at the two masses together with the known concentration of internal standard added to the plasma samples were used to calculate the concentration of thiothixene present.

A GC/CIMS-SIMS procedure was developed for the determination of thiothixene in plasma using a 3% SP-2250-DB column (sections 4.4, 5.7). The mass fragment at 113 was used for quantitation. As above, trideuterothiothixene was used as the internal standard. The percent of the inactive trans-isomer can also be determined using this procedure. The trans-isomer exhibits a mass fragment at m/e 447 which was not observed for the cis-isomer. Plasma samples were prepared by centrifugation of blood in a heparinized vacuum container. Four mL aliquots of plasma were taken, internal standard added, and the pH adjusted to pH 10.5-11.5 by dropwise addition of 1 N sodium hydroxide. The samples were then extracted with 6 mL of ether-hexane (3:1, v/v) followed by two 4 mL extractions using the ether-hexane mixture. The organic phases were combined and evaporated at 30°C under nitrogen. The residue was dissolved in 10  $\mu$ L of methanol; 5  $\mu$ L was used for the determination of thiothixene content in plasma. The detection limit was less than 1 ng/mL of plasma [36].

An HPLC procedure using a Varian Micropak CN 10 or Waters radial compression columns and ultraviolet or electrochemical detection has been described for the determination of thiothixene in plasma [60] (section 5.8). Plasma samples were prepared by alkalinizing 1 mL of plasma with 1 N sodium hydroxide and extracting this solution with mixed hexanes for

30 minutes. After centrifugation, a 9 mL aliquot of the hexane layer was removed and evaporated to dryness under a stream of nitrogen. The residues were redissolved in methanol (100  $\mu$ L) and 50  $\mu$ L was injected. Quantitation was accomplished by comparison with pure compound. Sensitivity was 0.1 ng/mL of plasma for ultraviolet detection and 0.01 ng/mL of plasma for electrochemical detection [55,60].

An additional HPLC method on a Hypersil Si 100 column (250 x 4.6 mm) using a mobile phase of 400 g water, 1000 g acetonitrile and 1.8 g tetraethylammonium perchlorate adjusted to pH 7.3 at a flow rate of 0.95 mL/min and a wavelength of 230 nm has been used for the detection of thiothixene in blood after extraction from 1 N sodium hydroxide solution into 1:1 hexane-ether; 0.1 N sulfuric acid was added to the organic phase. After extraction, 1:1 hexane ether and 1 N sodium hydroxide was added to the sulfuric acid. The mixture was vortexed and a portion of the organic layer was evaporated and reconstituted in mobile phase [61].

A fluorometric method using 310 nm activating and 440 nm fluorescent wavelengths and an Aminco Bowman spectrofluorometer was used to determine thiothixene in plasma [44,62,63]. Two mL of the plasma was alkalinized with 0.5 mL of 10 N sodium hydroxide and extracted with 10 mL of *n*-heptane in a 25 mL glass stoppered tube by shaking on an automatic shaker for 15 minutes [44] or 5 minutes [33]. After centrifuging for 5 minutes, the heptane phase was extracted using 1.5 mL of 0.1 N sulfuric acid [44] or 3 mL of 0.2 N sulfuric acid [33]. To 1 mL [44] or 3 mL [33] of the aqueous phase 0.5 mL [44,64] or 1 mL [33] of 2 M acetate buffer pH 5.5 was added. The sample was oxidized with 0.1 mL [44] or 0.2 mL [33] of 0.1% potassium permanganate solution. After 5 minutes, the excess potassium permanganate was reduced by adding 0.1 mL [44] or 0.2 mL [33] of 0.1% hydrogen peroxide. The fluorescence of the oxidized drug was then determined. The sensitivity was estimated at less than 1 ng without extraction (pure drug) and 3 ng when extracted from plasma. Fluorescence was linear to 500 ng/mL of plasma. *N*-Demethylthiothixene, a decomposition product can interfere with this assay. Variations in results for plasma samples have been reported [62] for this method. The solutions were excited at 280 nm and their relative emission fluorescence noted at 440 nm [33].

Plasma samples were also analyzed after extraction of 1-5 mL with phosphate buffer (pH 7.4, 0.1 M) and 10 mL of diethyl ether for 30 minutes followed by treatment of 9 mL of the ether layer with 1 mL of 1 N sodium hydroxide and

extraction with mixed hexanes for thirty minutes. After centrifugation, a 9 mL aliquots of the hexane layer was evaporated to dryness and redissolved in mobile phase [56]. HPLC was used in the analysis as described in section 5.8.

### 8.2 Blood

Blood samples can be analyzed using the HPLC procedure described in section 5.8 after treatment of 10 mL of whole blood with 1 mL of 1 N sodium hydroxide solution and extraction with two 10 mL portions of mixed hexanes for 30 minutes or one 15 mL aliquot of mixed hexanes for 1 hour. Measured aliquots of the hexane extracts were combined and evaporated to dryness. The residue was redissolved in 1 mL of 0.1 N hydrochloric acid and the compound of interest was extracted into 5 mL of chloroform by shaking gently for 10 minutes or by vortexing for 1 minute followed by centrifugation. A 4.5 mL aliquot of the chloroform layer was evaporated to dryness and redissolved in 10  $\mu$ L of mobile phase [56].

A fluorometric method using a Perkin Elmer MPF2A spectrofluorometer with a xenon light source was used to determine thiothixene in serum [64]. One mL of the serum was alkalized with 0.3 mL of 10 N sodium hydroxide, allowed to stand for 30 minutes, and vortexed for 1 minute with 6 mL of *n*-heptane containing 1.5% iso-amyl alcohol. After centrifuging for 10 minutes, 5 mL of the heptane phase was back extracted using 1.5 mL of 0.1 N sulfuric acid by vortexing for 1 minute, centrifuging for 10 minutes, and aspirating the heptane phase into waste. To 1.5 mL of sulfuric acid, 0.1 mL of acetate buffer (pH 5.0) was added. After mixing, 0.1 mL of 0.1% hydrogen peroxide was added. The maximum excitation was found at 385 nm. A slit width of 11 nm and an emission slit of 6 nm was used. The fluorescence was then determined at 445 nm.

### 8.3 Urine and Gastric Contents

The thiothixene content of urine and gastric contents can be determined using thin layer chromatography. Ten mL of urine or gastric contents were saturated with anhydrous potassium carbonate and shaken with 1 mL of ethanol. The ethanol was transferred to a 125 mL separatory funnel containing 50 mL of diethyl ether. The mixture was shaken and washed with pH 11 carbonate/bicarbonate buffer. The organic layer was centrifuged, separated from the aqueous layer, dried over anhydrous sodium sulfate, and evaporated under a stream of nitrogen. The residues were spotted in methanol using the systems described in section 5.6 [33].

#### 8.4 Other

A gas chromatographic procedure was used in the analysis of liver samples [49] as described in section 5.7. Thirty grams of liver were homogenized in 20 mL of water for 30 seconds. After the addition of 110 mL of ethanol the solution was homogenized for 2 minutes. After centrifugation, the weight of the separated supernatant was adjusted to 150 g by addition of ethanol and the extract was filtered. To 2.5 g of extract (0.5 g of wet liver tissue), 200  $\mu$ L of methanol solution was added containing 0.05 mg/mL of cyclizine and 0.1 mg/mL of mesoridazine as internal standards. The solution is evaporated to near dryness under a stream of nitrogen. The residue is reconstituted in 0.5 mL of Tris buffer yielding a final pH of 9.0. The mixture was shaken for 1 minute after the addition of 1 mL of butyl acetate. The butyl acetate layer is removed after centrifugation and injected into the gas chromatograph.

Thiothixene can be analyzed in brain tissue by the fluorescence assay used in plasma determinations [63] after treatment. The brain tissue is homogenized by hand in a mixture of 2 mL of 1 M sodium carbonate and 2 mL of 1 M sodium bicarbonate. The homogenate is extracted by shaking with 10 mL of 1,2-dichloroethane for 15 minutes. After centrifugation for 20 minutes, 5 mL of the dichloroethane phase was withdrawn. The remaining material was homogenized again and re-extracted with 10 mL of 1,2-dichloroethane for 15 minutes. The phases were again separated by centrifugation and 10 mL of the dichloroethane solution was removed and combined with the previous 5 mL aliquot. The thiothixene was then extracted into 1.5 mL of 0.1 M sulfuric acid by shaking for 15 minutes and determined by the fluorescence assay described previously.

#### 9. Determination in Pharmaceuticals

Thiothixene was analyzed on silica gel of controlled surface porosity bonded to a solid spherical core, 30  $\mu$ m to 50  $\mu$ m in diameter using a mobile phase prepared by mixing 1400 mL of ethanolamine-ethanol (0.5 mL to 3780 mL) with 200 mL of water. The column was maintained at ambient temperature. An ultraviolet detector at 254 nm is used for quantitation [22]. A weighed portion of thiothixene capsules equivalent to 10 mg of thiothixene was transferred to a 500-mL volumetric flask. After adding 400 mL of methanol and shaking for 10 minutes, the flask was placed in an ultrasonic bath for 5 minutes and then diluted to volume with methanol. The suspension was filtered through a 5- $\mu$ m polytetrafluoro-

ethylene membrane filter. A 20- $\mu$ L portion of this assay preparation and 20  $\mu$ L of a reference standard solution of thiothixene at similar concentration are concomitantly analyzed.

Thiothixene was analyzed using a Corasil II column and a mobile phase consisting of 0.37 mL of ethanolamine and 400 mL of water diluted to 2.8 mL with methanol. Ultraviolet detection at 254 nm was used. N-(1-naphthyl)ethylenediamine dihydrochloride was used as the internal standard [50]. An accurately weighed portion of thiothixene capsules equivalent to 25 mg of thiothixene was transferred to a glass-stoppered centrifuge tube and 5 mL of N-(1-naphthyl)ethylenediamine dihydrochloride solution and 20 mL of methanol was added. The resulting solution was shaken for 2 minutes and centrifuged. The supernate was used in the analysis. For thiothixene injection, an aliquot of sample equivalent to 25 mg of thiothixene was pipetted into a 25-mL volumetric flask and 5 mL of N-(1-naphthyl)ethylenediamine dihydrochloride solution was added. The solution was mixed and the flask was diluted to volume with methanol. Four  $\mu$ L of these assay preparations and a standard preparation at similar concentration were injected.

### References

1. Sterlin, C., Ban, T.A., et al., Curr. Ther. Res., 14(4), 205 (1972).
2. Barchas, J.D., Berger, P.A., et al., Psychopharmacology, Oxford University Press, New York (1977).
3. Koch, M.H.J., Mol. Pharmacol., 10, 425 (1974).
4. Gallant, D.M., Bishop, M.P., Curr. Ther. Res., 9(4), 153 (1974).
5. Abernathy, C.O., Ezekiel, M., et al., Arch. Int. Pharmacodyn., 234, 193 (1978).
6. Burger, A. Medicinal Chemistry, 32nd Edition, Wiley Interscience, New York (1970).
7. Weissman, A. Psychopharmacol., 12, 142 (1968).
8. Simeon, J., Keskiner, A., et al., Curr. Ther. Res., 9(1), 10 (1967).
9. Kurland, A.A., Alcides, P., et al., Curr. Ther. Res., 9(6), 298 (1967).
10. Brauzer, B., Goldstein, B.J., J. Clin. Pharm., 8, 400 (1968).
11. Holden, J.M.C., Itil, T.M., et al., Curr. Ther. Res., 13(5), 298 (1971).
12. Overall, J., et al., Clin. Pharm. Ther., 10(1), 36 (1968).
13. Enna, S.J., Bennett, J.P., et al., Nature, 263, 338 (1976).
14. Sugarman, A.A., Stolberg, H., Curr. Ther. Res., 7(5), 310 (1965).
15. Ban, T.A., Lehmann, H.E., Dis. Nerv. System, 36(9), 473 (1975).
16. Iversen, L.L., Sci., 188, 1084 (1975).
17. Bunney, B.S., Walters, J.R., et al., J. Pharmacol. Expt. Ther., 185(3), 560 (1973).

18. Weisel, F.A., Bjerkenstedt, L., et al., Acta Pharmacol. Toxicol., 43, 129 (1978).
19. Wyatt, R.J., Psychopharmacol. Bull., 12(3), 5 (1976).
20. Bjerkenstedt, L., Gullberg, L.B., Arch. Psychiat. Neurol. Sci., 224, 107 (1977).
21. Sedvall, G., Bjerkenstedt, L., Life Sci., 23, 425 (1978).
22. The United States Pharmacopeia, 21st revision, Mack Publishing Company, Easton, Pennsylvania (1985) p. 1057 and Eighth Supplement (1988) p. 3057.
23. The Merck Index, Tenth Edition, Merck & Co., Inc., Rahway, New Jersey (1983) p. 1342.
24. USP DI. Drug Information for the Health Care Professional, Ninth Edition (1989) Vol. IB, p. 2319.
25. Clarke, E.G.C., Isolation and Identification of Drugs, 2nd Edition, The Pharmaceutical Press, London, (1985) p. 1021.
26. The United States Pharmacopeia, op. cit., p. 1479.
27. USAN and the USP Dictionary of Drug Names, United States Pharmacopeial Convention, Inc., Rockville, Maryland (1989) p. 547.
28. Muren, F., Bloom, B.M., J. Med. Chem., 13, 17 (1970).
29. Hobbs, D.C., J. Pharm. Sci., 57(1), 105 (1968).
30. Mills, T., Price, W.N., et al., Instrumental Data for Drug Analysis, Vol. 1, Elsevier, New York (1982) p. 568.
31. Wyatt, D.K., A Study of Selected Dibenzocycloheptane and Thioxanthene Derivatives by Carbon-13 Nuclear Magnetic Resonance Spectroscopy, University Microfilms International, Ann Arbor, Michigan (1985).
32. Auterhoff, H., Kuhn, J., Arch. Pharm., 306(4), 241 (1973).
33. Kemal, M., J. Anal. Toxicol., 9, 94 (1985).



34. McLinden, V.J., Stenhouse, A.M., Foren. Sci. Int., 13, 71 (1979).
35. Hobbs, D.C., Clin. Pharmacol. Ther., 16(3), 473 (1974).
36. Bombardt, P.A., Friedel, R.O., Commun. Psychopharmacol., 1, 49 (1977).
37. Irwin, W.J., Po, L.W., Proc. Analyt. Div. Chem. Soc., 329 (1979).
38. Po, L.W., Irwin, W.J., J. Pharm. Pharmacol., 31, 512 (1979).
39. Po, L.W., Irwin, W.J., J. Pharm. Pharmacol., 32, 25 (1980).
40. Schaeffer, J.P., Chem. Commun., 743 (1967).
41. Tollenaere, J.P., Moereels, H., et al., Eur. J. Med. Chem., 12(3), 199 (1977).
42. Gifford, L.A., Miller, J.N., et al., Talanta, 24, 273 (1977).
43. Gantes, P., Barat, J. et al., Annal. Pharm. Franc., 27(11), 645 (1969).
44. The National Formulary, 14th revision, Mack Publishing Company, Easton, Pennsylvania (1985) p. 711.
45. Mjorndal, T., Orelund, L., Acta Pharmacol. Toxicol., 29, 295 (1971).
46. Kroger, V.H., Bohn, G., Rucker, G., Dtsch. Apoth. Ztg., 117, 1923 (1977).
47. Dobrecky, J., Rev. Farm. (Buenos Aires), 114, 42 (1972).
48. Kaniewska, T., Wejman, W., Farm. Pol., 30(8), 763 (1974).
49. Eklund, A., Jonsson, J., Schuberth, J., J. Anal. Toxicol., 7, 24 (1983).
50. Wong, C.J., Cohen, D.M., J. Pharm. Sci., 65(7), 1090 (1976).

51. Bogema, S.C., Narasimhachari, N. et al., J. Chromatogr., 233, 257 (1982).
52. Narasimhachari, N., Dorey, R.C. et al., J. Chromatogr., 311, 257 (1984).
53. Severin, G., J. Pharm. Sci., 76(3), 231 (1987).
54. Curry, H., Brown, E.A., IRCS Med. Sci., 9, 166 (1981).
55. Ibid, 9, 169 (1981).
56. Curry, S.H., Brown, E.A., et al., J. Chromatogr., 231, 361 (1982).
57. Wheals, B.B., J. Chromatogr., 277, 65 (1980).
58. Kaferstein, V.H., Sticht, G., Beitr. Gerichtl. Med., 41, 95 (1983).
59. Biden, L.E., Ruth, J.A., Experientia, 34(8), 1062 (1978).
60. Curry, H., Brown, E.A., IRCS Med. Sci., 9, 170 (1981).
61. Dilger, C., Salama, Z., Jaeger, H., Arzneim-Forsch/Drug Res., 38(II, 10), 1522 (1988).
62. Jacobsson, L., von Knorring, et al., Int. Pharmacopsy-chiat., 11, 206 (1976).
63. Mjorndal, T., Wiesel, F.A., Orelund, L., Acta Pharm. Toxicol., 38(5), 490 (1976).
64. Yesavage, J.A., Holman, C.A., Cohn, R., Psychopharm., 74, 170 (1981).
65. Dobrecky, J., Gonzalez, B., Rev. Farm. (Buenos Aires), 116, 60 (1974).

*Analytical Profile of D-Cycloserine*

*Humeida A. El-Obeid and  
Abdullah A. Al-Badr*

*Department of Pharmaceutical Chemistry,  
College of Pharmacy, King Saud University,  
Riyadh, Saudi Arabia.*

## Contents

### 1. Description

- 1.1 Nomenclature
- 1.2 Formulae
- 1.3 Molecular Weight
- 1.4 Elemental Composition

### 2. Physical Properties

- 2.1 Appearance, Color and Odor
- 2.2 Melting Range
- 2.3 Solubility
- 2.4 Optical Rotation
- 2.5 Spectral Properties

### 3. Synthesis

### 4. Biosynthesis

### 5. Stability

### 6. Methods of Analysis

- 6.1 Titrimetric Methods
- 6.2 Spectrophotometric Methods
- 6.3 Photographic Methods
- 6.4 Differential Thermal Analysis
- 6.5 Chromatographic Methods
- 6.6 Biological Methods

### 7. Pharmacokinetics

### Acknowledgment

### References

## 1. Description

### 1.1 Nomenclature

#### 1.1.1 Chemical Names

D-4-Amino-3-isoxazolidinone  
D-4-Amino-3-isoxazolidone

#### 1.1.2 Generic Names

Orientomycin, PA-94, 106-7, Closina, Far-  
miserina, Micoserina, Oxamycin, Seromycin (1).

#### 1.1.3 Trade Names

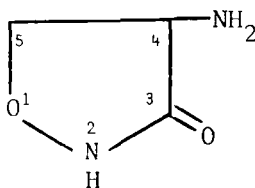
Aristoserina, Cichovalidin, Closina, Far-  
miserina, Miroseryn, Oxamycin, Serociclina, Seromycin,  
Setavax, Tisomycin (2).

### 1.2 Formulae

#### 1.2.1 Empirical

$C_3H_6N_2O_2$

#### 1.2.2 Structural



#### 1.2.3 CAS Registry No.

[68-41-7]

#### 1.2.4 Wiswesser Line Notation

T50MVTJ DZ \*DX (3)

### 1.3 Molecular Weight

102.09 (1)

### 1.4 Elemental Composition

C 35.29%, H 5.92%, N 27.44%, O 31.34% (1).

## 2. Physical Properties

### 2.1 Appearance, Color and Odor

Cycloserine occurs as a hygroscopic, white to pale yellow crystalline powder which may have a faint odor (4).

### 2.2 Melting Range

155-156°C [with decomposition] (1,3).

### 2.3 Solubility

Cycloserine is soluble in water. Aqueous solution has a pH around 6. It is slightly soluble in methanol and propylene glycol (1).

### 2.4 Optical Rotation

$[\alpha]_D^{23} + 116$  ( $c = 1.17$ ) (1,3).  
 $[\alpha]_{546}^{25} + 173$  ( $c = 5$  in 2 N NaOH) (1).

### 2.5 Spectral Properties

#### 2.5.1 Ultraviolet (UV) Spectrum

The UV absorption spectrum of D-cycloserine in neutral methanol is obtained on a Cary 219 spectrophotometer. The spectrum, shown on Figure 1, is characterized by a maximum at 215 nm. Reported UV maxima are 226 nm  $E(1\%, 1\text{cm})$  402 in water (1,3), 217 nm in 0.1 N HCl (3) and 222 nm in 0.1 N NaOH (3).

#### 2.5.2 Infrared (IR) Spectrum

The IR absorption spectrum of D-cycloserine is obtained from a potassium bromide diper-

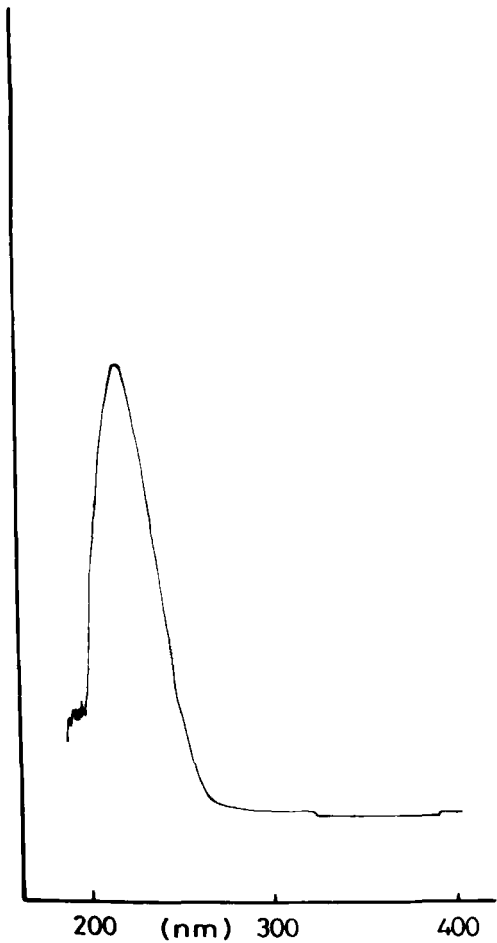


Figure 1: Ultraviolet spectrum of D-cycloserine in neutral methanol.

sion and is presented in Figure 2. The spectrum was recorded on a Pye Unicam SP 1025 IR spectrophotometer. The characteristic bands and their assignments are listed in Table 1. D-cycloserine in the solid state was shown to possess two ionizable groups with  $pK_1 = 4.4 - 4.5$  and  $pK_2 = 7.4$  (5-7). Formation of a zwitterion and a resonance stabilized hydroxamate anion is evident from the spectrum of cycloserine in Figure 2.

Table 1. Assignments of cycloserine characteristic IR peaks

<u>Frequency (cm<sup>-1</sup>)</u>	<u>Assignment</u>
3300-2100	Broad (-NH <sub>3</sub> ) absorption = N - H stretch, extended by a combination bands.
1630	Asymmetric (-NH <sub>3</sub> ) N-H band.
1600-1500	Absorption resonance stabilized hydroxamate anion.
800-650	N - H out of plane stretch.
500	Tortional (-NH <sub>3</sub> ) N - H oscillation

### 2.5.3 <sup>1</sup>H-Nuclear Magnetic Resonance (<sup>1</sup>H-NMR) Spectrum

The <sup>1</sup>H-NMR spectrum of cycloserine in D<sub>2</sub>O is obtained on a Varian XL-200 spectrometer and is shown in Figure 3. The assignments of the triplet centered at 4.23 ppm and the multiplet centered at 4.42 ppm are presented in Figure 3.

### 2.5.4 <sup>13</sup>C-Nuclear Magnetic Resonance (<sup>13</sup>C-NMR) Spectrum

The <sup>13</sup>C-NMR spectrum of cycloserine in D<sub>2</sub>O using dioxane as an internal reference is obtained on a Varian XL-200 spectrometer and is presented in Figure 4. The assignments of the chemical shifts (Figure 4) is based on APT and DEPT procedures.



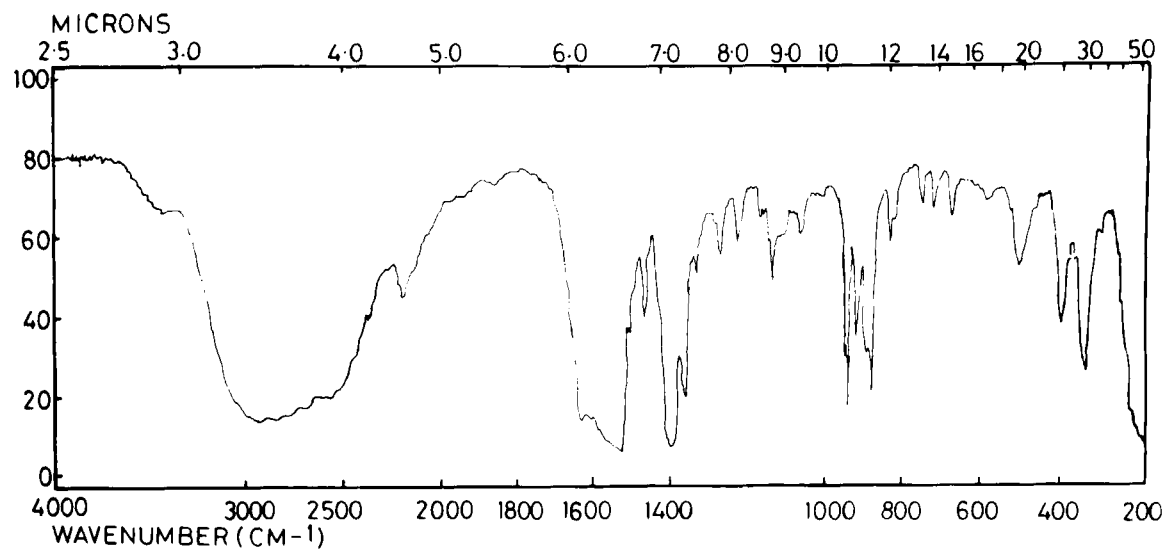


Figure 2: Infrared spectrum of D-cycloserine from KBr disc.

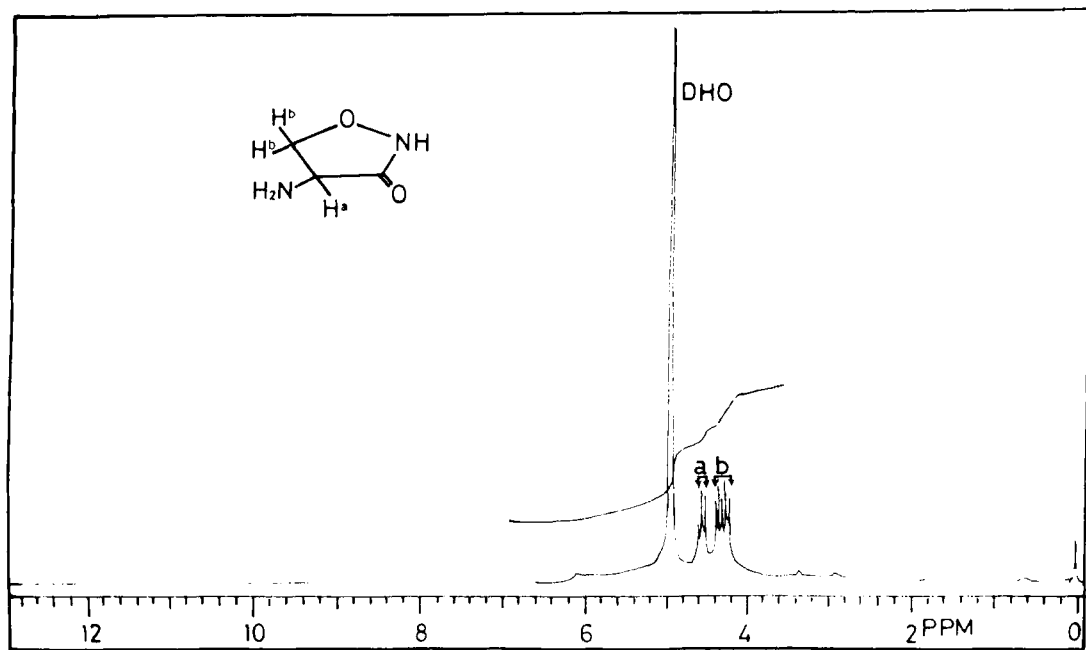


Figure 3:  $^1\text{H}$ -NMR spectrum of D-cycloserine in  $\text{D}_2\text{O}$ .

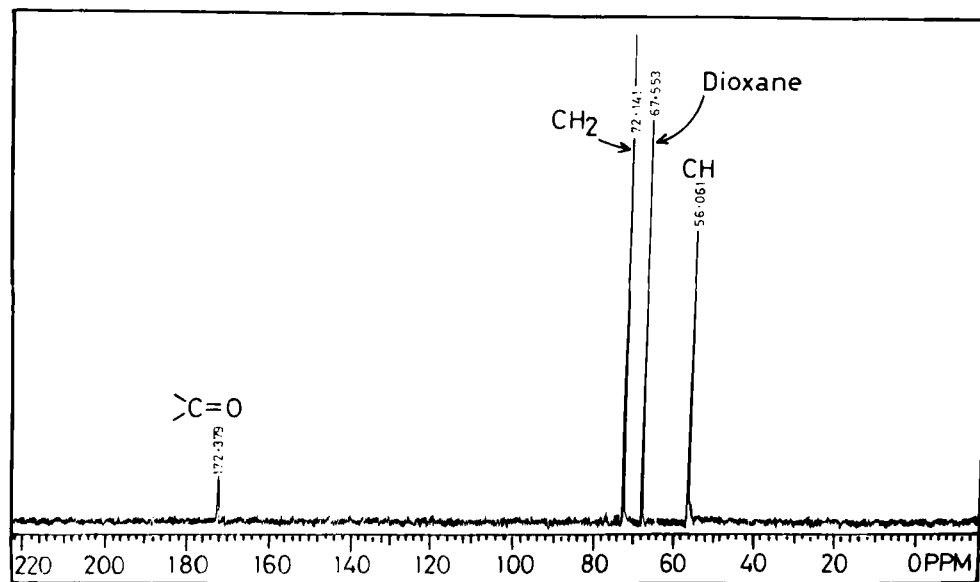
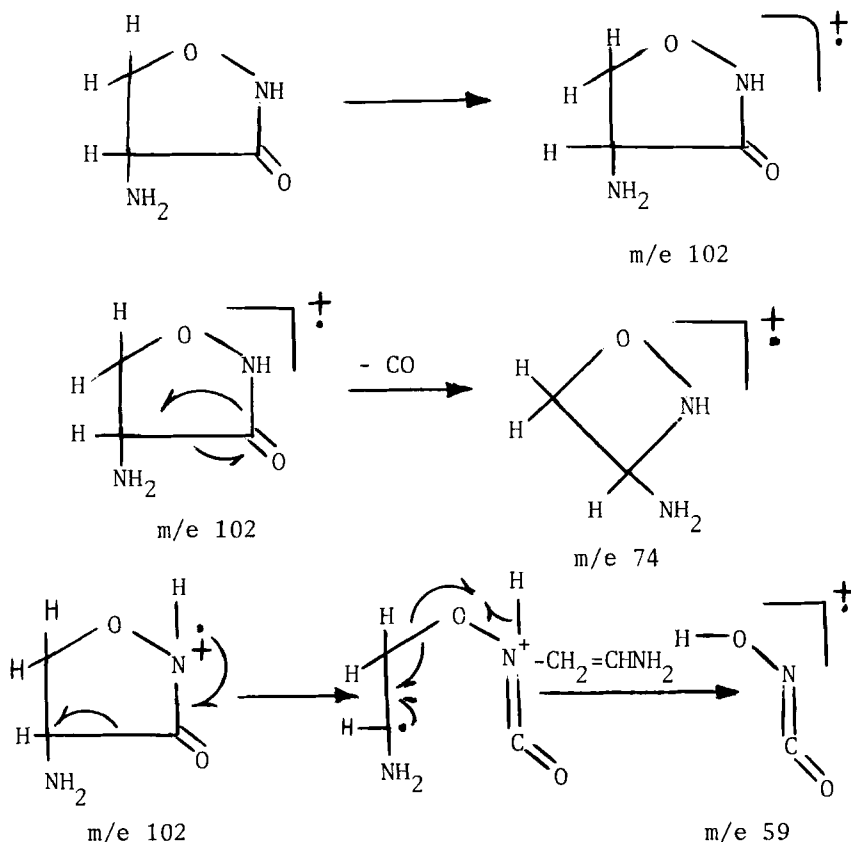


Figure 4:  $^{13}\text{C}$  NMR spectrum of D-cycloserine in  $\text{D}_2\text{O}$  with dioxane as internal reference.

### 2.5.5 Mass Spectrum

The literature reported no useful mass spectrum of D-cycloserine. However, Fuckushima and Arai (8) measured the field desorption mass spectrum for amino acid-related antibiotics including cycloserine.

In Figure 5 we present the mass spectrum of D-cycloserine obtained on a Finnigan Mat 4615 B mass spectrophotometer. The spectrum shows a molecular ion peak at  $m/e$  of 102 with a relative intensity of 33.15%, and a base peak at  $m/e$  of 59. A prominent diagnostic ion is observed at  $m/e$  74. The proposed fragmentation pattern leading to these ions is outlined below:



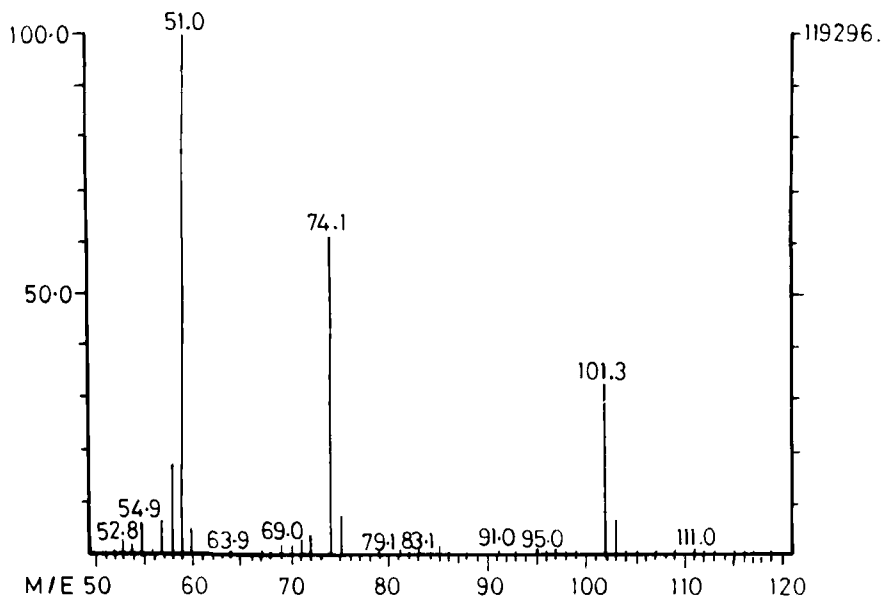


Figure 5: Mass spectrum of D-cycloserine

### 2.5.6 Thermal Analysis

Lamb (9) carried out differential thermal and thermogravimetric analyses on D-cycloserine. A melting endotherm was observed followed by a rapid exotherm. At heating rate of 20°C/min the endotherm peaked at 152°C and the exotherm at 160°C. The thermogravimetric analysis showed a 1.0% weight loss at 147°C. The weight loss rate increases rapidly as the melting point is approached.

We performed the thermal analysis of D-cycloserine on Du pont (TA 9900) DSC unit between 100°C to 200°C and 10°C/min heating rate (Fig. 6). Figure 6 shows that D-cycloserine decomposes before melting. The decomposition starts at 144°C and ends at 176°C with a maximum rate at 149.7°C. The decomposition occurs in two steps as shown by the first derivative of DSC curve and the overall heat of decomposition equals to 1440 J/gm (35.12 Kcal/mole).

### 2.5.7 X-Ray Powder Diffraction of D-Cycloserine

The x-ray diffraction pattern of D-cycloserine was determined with a Philips Pull automated X-ray Diffraction Spectrogoniometer equipped with PW 1730/10 generator. Radiation was provided by a copper target (Cu anode 2000 W,  $\gamma = 1.5480 \text{ \AA}$ ) and high intensity x-ray tube operated at 40 KV and 35 MA. The monochromator was a curved single crystal one (PW1752/00) Divergence slit and the receiving slit wire 1 and 0.1° respectively. The scanning speed of the goniometer (PW1050/81) used was 0.02-20 per second. The instrument is combined with Philips PM 8210 printing recorder with both analogue recorder and digital printer. The goniometer was aligned using silicon sample before use.

The x-ray pattern of D-cycloserine is presented in Figure 7. The interplanar distance  $d\text{\AA}$  and relative intensity  $I/I_0$  are shown in Table 2.

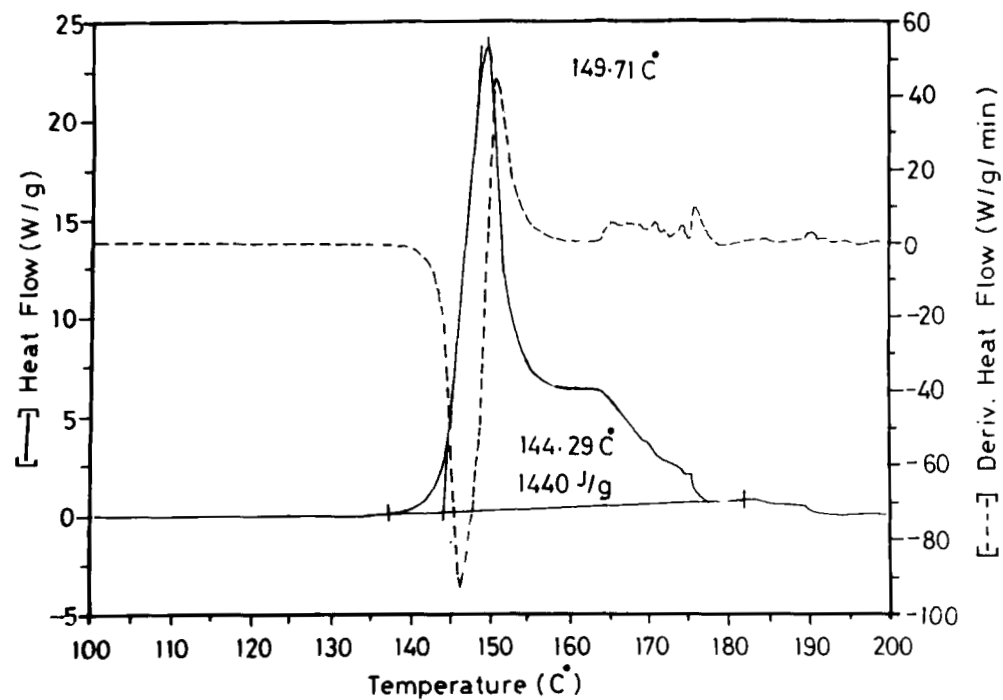


Figure 6: Thermal curve of D-cycloserine.

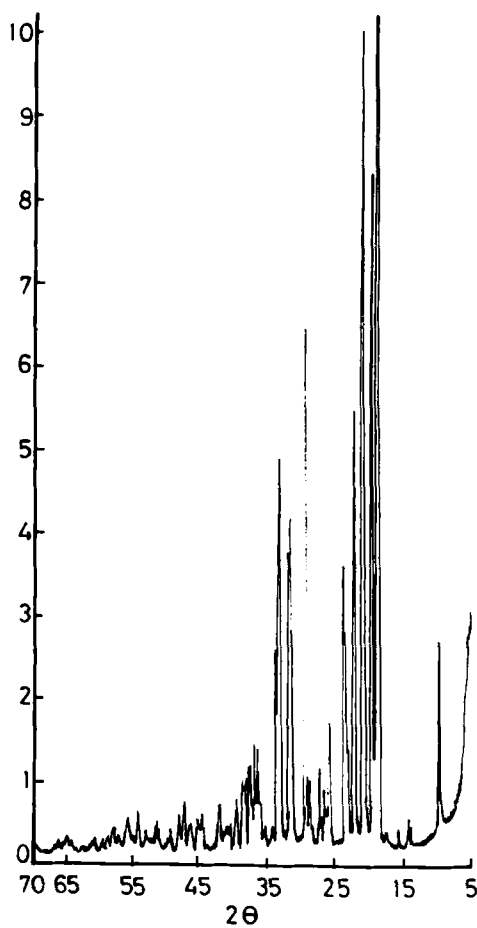


Figure 7: The X-ray diffraction pattern of D-cycloserine.



Table 2. X-Ray Diffraction Pattern of D-Cycloserine

d(Å)	I/I <sub>0</sub>	d(Å)	I/I <sub>0</sub>
16.53	20.25	2.18	3.39
9.10	18.48	2.15	2.91
6.21	3.95	2.13	5.36
5.67	3.30	2.06	2.01
5.10	2.98	2.02	4.15
4.71	100	2.00	4.13
4.55	57.80	1.95	3.79
2.24	70.16	1.92	5.26
4.00	37.82	1.89	3.75
3.84	12.17	1.84	3.05
3.71	23.23	1.82	2.02
3.44	11.90	1.77	3.07
3.39	6.08	1.77	3.46
3.34	6.27	1.73	2.33
3.25	8.37	1.72	2.97
3.10	7.74	1.69	4.60
3.02	43.76	1.66	2.54
2.82	28.46	1.64	3.47
2.69	33.73	1.64	4.01
2.67	14.14	1.60	2.64
2.62	3.00	1.58	3.22
2.54	3.24	1.57	2.76
2.49	5.45	1.55	2.22
2.46	9.59	1.52	2.46
2.43	10.27	1.49	1.98
2.40	8.58	1.48	1.83
2.35	7.32	1.45	2.03
2.27	5.54	1.43	2.56
2.26	4.43	1.42	2.23
2.22	3.66	1.40	2.19
2.20	3.30		

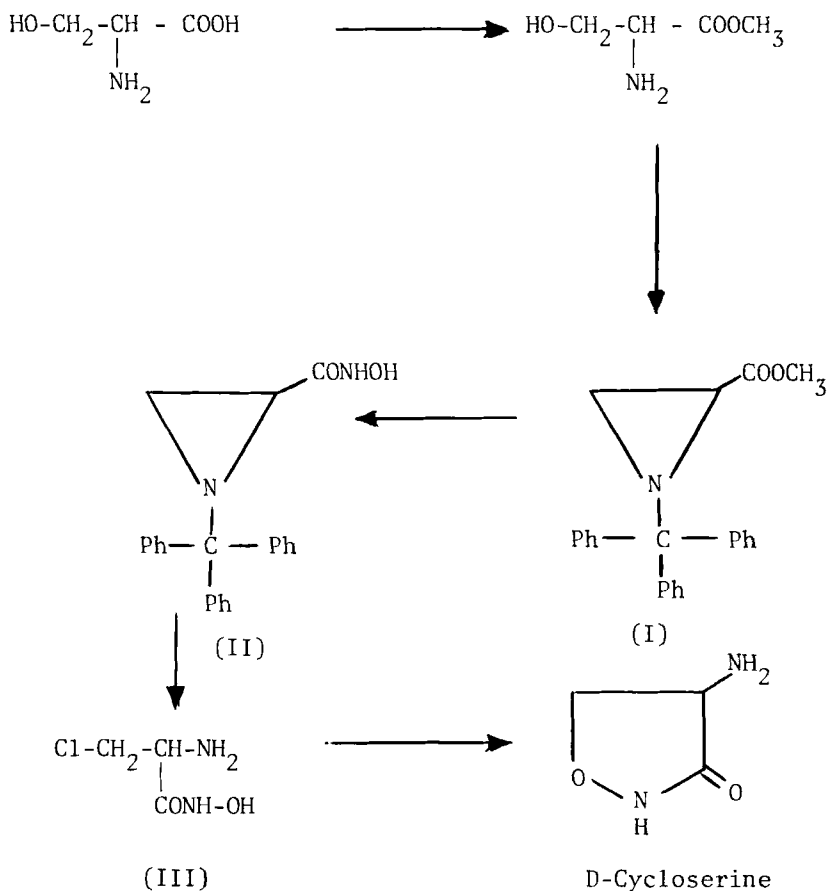
d = interplanar distance, I/I<sub>0</sub> = relative intensity (based on highest sensitivity of 100).

### 3. Synthesis

D-Cycloserine can be chemically synthesized by the method of Evan (10) in which DL-serine is converted to

its methyl ester. The ester is treated with triphenylmethyl chloride and methane sulfonyl chloride to give the substituted ethylene amine (I, Scheme I). The latter (I) can be converted into the hydroxamic acid (II). Reaction of (II) with hydrochloric acid yielded D- $\alpha$ -amino- $\beta$ -chloro-N-hydroxypropionamide (III) which upon treatment with a basic ion exchanged cyclizes to D-cycloserine.

Scheme I. Synthetic route of D-cycloserine



#### 4. Biosynthesis

D-Cycloserine is produced by various *Streptomyces*. Harned et al. (11) isolated the antibiotic from culture filtrates by adsorption onto a strong base anion exchange resin and elution with  $\text{H}_2\text{SO}_4$ . The antibiotic is then converted to a water-insoluble silver salt. The isolated pure salt is then decomposed with  $\text{HCl}$  to give cycloserine which is crystallized from the filtrate with alcohol or acetone.

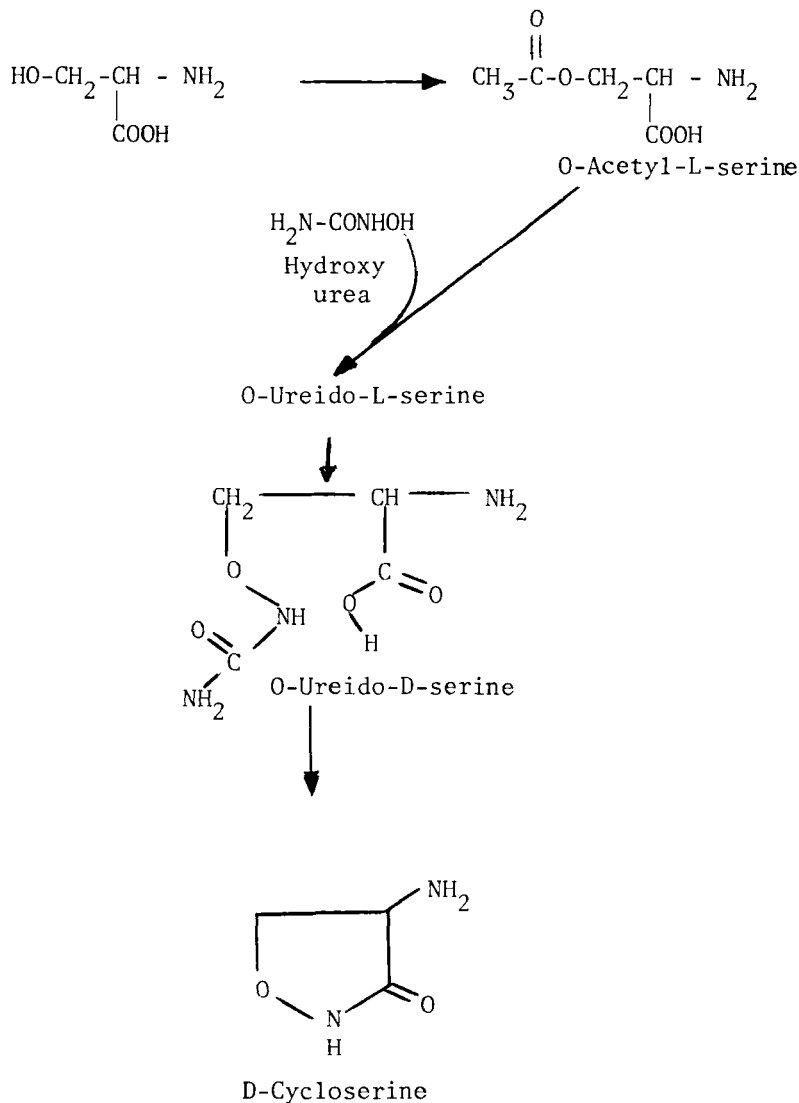
A more recent method of isolation of crystalline D-cycloserine from fermentation broth filtrates is reported by Yakhontova et al. (12). The method includes sorption of cycloserine in the cationic form by a strong cross-linked sulfo-cation exchange resin (on the basis of styrene and divinylbenzol) in a series of columns. Desorption is accomplished using an aqueous ammonia solution. A product of high purity is claimed to be obtained from the diluates after their clarification, evaporation and dilution of the concentrate with ethyl alcohol.

Cai et al. (13) presented evidence for the presence of plasmids in *Streptomyces* and their possible control over the biosynthesis of antibiotics. They carried out curing studies with acridine orange and indicated that the production of antibiotics by three strains of *Streptomyces* was plasmid-determined, with curing frequency of 5-10%. Curing of *Streptomyces* strain 2286 with acridine orange caused loss of the ability of synthesizing cycloserine, accompanied by the disappearance of the intermediate O-carbamyl-D-serine.

Using incubation experiments, Svensson and Gatenbeck (14) proposed a pathway for the biosynthesis of D-cycloserine in *Streptomyces garyphalus*. The incubation experiments carried out with washed cells and toluene-treated cells of *S. garyphalus*, showed that O-acetyl-L-serine and hydroxyurea are intermediates in the biosynthesis of D-cycloserine. The formation of [ $^{14}\text{C}$ ]O-ureidoserine from O-acetyl-L-serine and hydroxyurea was demonstrated by enzymic incubations using  $^{14}\text{C}$ -labeled substrates. Desalted cell-free extracts catalysed the conversion of O-ureido-D-serine to D-cycloserine in an ATP- and  $\text{Mg}^{++}$ -requiring reaction. The proposed path-

way for D-cycloserine biosynthesis is: L-serine  $\rightarrow$  O-acetyl-L-serine  $\rightarrow$  O-Ureido-L-serine  $\rightarrow$  O-Ureido-D-serine  $\rightarrow$  D-cycloserine (Scheme II).

Scheme II. Pathway of the biosynthesis of D-cycloserine



## 5. Stability

Cycloserine deteriorates upon absorbing water and is destroyed at neutral or acidic pH. D-cycloserine capsules should be stored in tight containers at less than 40°C, preferably between 15-30°C (4).

Absorbed moisture from the air was found to affect the stability of cycloserine preparations in the metal packed capsules produced by various companies after testing for more than one year (15).

Ciestak et al. (16) studied the effect of acidity on the stability of cycloserine during its recovery from filtered culture broth. When an aqueous solution of cycloserine adjusted to pH 1.26-3.60, the degree of decomposition of the drug was 50% after 22 hrs of standing at 25°, and 25% at 4°, irrespective of the concentration or type of acid used. The product of decomposition was identified as  $\beta$ -aminooxalanine which is believed to be subsequently converted to serine.

The stability of samples of aluminium foil sealed cycloserine capsules produced by different companies were tested at room temperature, 37° and 38° with saturated humidity. Samples from the company kept under the last condition, decomposed after one month storage (17).

Kartseva et al. (18) studied the stability of  $\alpha$ -cycloserine samples containing various amounts of moisture and kept in sealed glass tubes with silica gel at one end. The samples were analysed when the controls without silica gel reached 20% decomposition. At 40° the greater the extent of decomposition. Little decomposition was observed at 0.15% moisture. However, at 1.3% a sharp drop was observed with increasing distance from the silica. When the powder with 1.1% moisture was initially stored for 12 days at 5-8° or 18-20° and then at 40°, little decomposition was observed.

## 6. Methods of Analysis

### 6.1 Titrimetric Methods

#### 6.1.1 Non-Aqueous Titration

Cycloserine was determined (19) by non-aqueous titration using the following procedure:

Dissolve 0.1 g of active substance, or the equivalent amount of powdered tablet, in 15 ml of conc. acetic acid, add 30 ml of dioxan and 4 drops of 1% methanolic mentanil yellow (C.I. Acid yellow 36), and titrate with 0.1 N perchloric acid in dioxan to the color change from yellow to red-violet; 1 ml of acid = 10.2 mg of cycloserine.

#### 6.1.2 Potentiometric Methods

Braibante et al. (20) studied the equilibrium of D-cycloserine with protons and cobalt (II), nickel (II), copper (II) zinc (II) aqueous ions in solutions, the equivalent of D-cycloserine (HL) with the ions of H, Co, Ni, Cu and Zn were studied potentiometrically at 25° and 0.1 mol/dm<sup>3</sup> KCl. The protonation constants are  $\log K = 7.346(5)$  ( $-\text{NH}_3^+$ ) and  $\log K_2 = 4.388(6)$  ( $-\text{OH}$ ); the corresponding enthalpy changes are - 32.25(15) and - 14.52(15) KJ/mol respectively. The metal ions form the complexes  $\text{M}(\text{HL})^{2+}$ ,  $\text{ML}^+$  and  $\text{ML}_2$ . Stability contents are given.

### 6.2 Spectrophotometric Methods

Several spectrophotometric methods for the assay of cycloserine were reported. Kartseva and Bruns (21) modified the method described by Jones (22) to avoid errors caused by the change in extinction of test solution with time when fresh reagent is used. With the reagent (4%  $\text{Na}_2\text{Fe}(\text{CN})_5 \cdot \text{NO} \cdot 2\text{H}_2\text{O}$  solution - 4N-NaOH (1:1) stored for 24 hours in a refrigerator before use, the extinction remains almost constant for 6 to 8 hours. The test solution (1 ml containing 50 to 200  $\mu\text{g}$  of cycloserine per ml) is added to N-acetic acid (3 ml) and 1 ml of reagent; after 10 minutes, the extinction of the blue solution is measured (5 mm all; red filter). Results are reproducible to within  $\pm 1\%$ .

The determination of cycloserine in blood was reported by Svinchuk et al. (23). A 2 ml blood sample was mixed with 1 ml of 20% trichloroacetic acid and the ppt. was removed by filtration on a glass filter. The precipitate was washed with 1 ml water and the filtrate was treated with alkaline sodium bicarbonate solution (5 g NaOH + 7.2 g NaHCO<sub>3</sub> in 250 ml water) until it reached pH 6, then 0.3 ml 5% ferric oxide nitrate in 1% nitric acid was added, the solution was made up to 5 ml, and after 5 minutes the absorbance was measured at 400 nm. The standard curve of cycloserine in blood was linear from 0.05 & 0.5 mg/sample.

Svinchuk et al. (24) determined cycloserine in other biological materials, gastric juice, urine, blood and plasma of patients who had received cycloserine, were treated with trichloroacetic acid and filtered. In each instance, the pH of the filtrate was raised to 6 with NaOH-NaHCO<sub>3</sub>, Fe(NO<sub>3</sub>)<sub>3</sub> solution in nitric acid was added, and the absorbance was measured at 400 to 480 nm.

Svinchuk (25) also determined cycloserine, isoniazid and ethionamide. The sample (20 ml) was treated with 3 ml of 20% trichloroacetic acid solution and centrifuged or filtered. The drugs are determined in 5 ml aliquots of the solution. The aliquot for cycloserine is treated 5% Fe(NO<sub>3</sub>)<sub>3</sub> solution, in 1% nitric acid and then with ethyl ether and the absorbance of the aqueous layer is measured at 400 nm vs a blank containing isoniazid and ethionamide. A calibration graph is prepared from solution containing the other two analytes.

Hiremath and Mayanna (26) described a colorimetric method for the determination of D-cycloserine in tablet and capsules.

Spectrophotometric and spectrofluorometric methods were reported (27) for the determination of cycloserine; both methods were based on the reaction of the antitubercular in aqueous solution of pH 8.2 with p-benzoquinone. Beer's law was obeyed over the concentration range of 4-20 µg/ml for the spectrophotometric method and 0.04-0.2 µg/ml for the fluorometric method.

The analysis of D-cycloserine in solutions, tablets and capsules, based on oxidative reaction with chloramine-T was reported (28).

An automated method for assay of cycloserine in fermentation broths was presented (29). The method is based on the determination of the colored complex between cycloserine and sodium nitroprusside. It is effective for concentrations ranging from 50-500  $\mu\text{g/ml}$  and agrees rather well with manual and microbiological methods. The maximum analytical error was 2.5%.

### 6.3 Photographic Methods

Chemiluminescence determination of micro amounts of organic reductants (drugs including cycloserine) by reaction involving vanadate (30). The method involves reduction of 0.5 m  $\text{M-NaVO}_3$  to  $\text{V}^{1\text{v}}$  by an organic reductant in 0.05 N -  $\text{H}_2\text{SO}_4$  medium. The reaction mixture is heated for 10 to 40 minutes and the  $\text{V}^{1\text{v}}$  is determined photographically by its catalysis of the chemiluminescence reaction is carried out with 20  $\mu\text{M}$ -luminol in sodium carbonate-sodium bicarbonate buffer solution of pH 11. The method was used for determining 0.5 to 3  $\mu\text{g}$  in 5 ml solution of cycloserine. The coefficient of variation were < 30%.

A photoelectrocolorimetric method for the quantitative determination of cycloserine, isoniazide and ethionamide in urine was described (31). The method can be used without preliminary separation of the three compounds, even if they are simultaneously present in the urine.

### 6.4 Differential Thermal Analysis

Mohamed and Tawakkol (32) have reported a quantitative differential thermal analysis (DTA) of cycloserine. The drug was detected in bulk powder and tablets by a DTA method based on the electric voltage generated by a thermocouple due to a difference in temperature ( $\Delta T$ ) between the sample and a reference material for the time interval during which the phase change occurs as the system is heated. An empty aluminium crucible was the reference material and the heating rate maintained at 5% minutes. Areas under the



$\Delta T$ -time curve were obtained and the relation between the peak area and the mass of cycloserine was linear at 1-5 mg/ cycloserine. The recovery was 99.1% and the accuracy of the method 99.1%.

## 6.5. Chromatographic Methods

### 6.5.1 Thin-Layer Chromatography (T.L.C.)

Rapid identification (33) of cycloserine and other most frequently used antibiotics is possible by thin-layer chromatography on silica gel G using a 5:1 mixture of 10%  $\text{CuSO}_4$  and 2%  $\text{NH}_4\text{OH}$  as spotting agent. In a mixture of 8:1:1 of 3.9% ammonium hydroxide 10% acetic acid and acetone, the  $R_f$  value of cycloserine was 0.9 (green spot). In a mixture of 5:1:3:1 of propanol-ethyl acetate-water and 25% of ammonium hydroxide,  $R_f$  value of cycloserine was 0.55 (green spot).

Voigt and Maa Bared (34) reported the use of thin-layer chromatography for the separation and identification of six tuberculostatic antibiotics. The drugs were separated on plates coated with a dispersion of Kieselgel G in isopropanol and activated at  $105^\circ$  for 30 minutes. Two dimensional development is carried out in: (a) acetone-2% aqueous sodium acetate (9:1) for 40 minutes and (b) butanol-pyridine-methanol-anhydrous acetic acid water (30:20:20:1) for 130 minutes. The zone containing cycloserine was sprayed with a mixture of (1:1) solution of 500 mg of p-dimethyl amino benzaldehyde in 50 ml of cycloserine and 200 mg of ninhydrin in 50 ml of 95% ethanol. When the plate is heated at  $100^\circ$  for 60 to 90 seconds, characteristic colors, stable for several hours are given for by cycloserine and other antibiotics; optimum amount for detection are 2 to 5  $\mu\text{g}$ .

### 6.5.2 Paper Electrophoresis

Garber and Dobrecky (35) reported a study of antibiotics by paper electrophoresis and identified six antibiotics using three different solvent systems: 1, 5 and 10% aqueous ammonia. Whatman No. 1 paper was used as a support. Cycloserine moved to the anode and the others moved to the cathodes. In general, the

electrophoretic mobility decreased with increasing ammonia concentration. A development time of 2 hours allowed the separation of a mixture of the six antibiotics.

#### 6.5.3 Gas-Liquid Chromatography (GLC)

David et al. (36) described the following glc system:

Prepare an extract of the sample (cycloserine) in pyridine-water (2:1), evaporate to dryness 1 ml of the solution containing 1 mg of cycloserine, and heat the residue at 80° for 10 minutes, with 0.1% hexamethylbenzene solution in chloroform (1 ml). NO-bis(trimethylsilyl) acetamide (100 µl) and trimethylchlorosilane (10 µl) in a closed vessel. Submit 1 to 5 µl of the product to g.l.c. on a glass column (3 f X 0.25 in. o.d.) containing 3.8% of UCW-98 on Diatoport S (80 to 100 mesh), operated at 115° with helium as carrier gas (55 ml per minute) and flame ionization detection; measure the peak areas. The coefficient of variation (5 determinations) was 0.5%. Cycloserine dimer, 3,6 dimethylenepiperazine-2,5 dione and 3-aminoxalanine do not interfere.

#### 6.5.4 High Performance Liquid Chromatography (HPLC)

Musson et al. (37) described an ion-pair reverse phase HPLC assay for the simultaneous quantitation of cycloserine and its prodrug, pentizidone in human plasma and urine.

### 6.6 Biological Methods

Cycloserine was detected in food and was estimated by a simplified zone inhibition method. The bioassay cycloserine and other antibiotics in food was carried out using bacteria (38).

The calculation of the kinetics of extraction of cycloserine and other antibiotics from the native solutions by a fluidized-bed ion-exchange method, have been reported (39). A mathematical formula and a nanogram for the kinetics of extraction of antibiotics from native solutions by the title method were suggested. The

calculated results were in good agreement with experimental data e.g. on the extraction of cycloserine and of Kanamycin.

The microbiological turbidimetric potency assay for cycloserine and other antibiotics is modified (40) under the federal food drug and cosmetic act, to provide for developing a standard curve with concentrations of 64, 80, 100, 125 and 156% of the reference concentration of the assay. More accurate potency concentration estimates are obtained when samples are diluted to a concentration in the 80-125% range. The modification are for tests and methods of assay of antibiotics and antibiotic-containing drugs; revised standard response line concentrations.

An indicator method of determining tuberculostatic drugs (including cycloserine) in the urine was published (41). An indicator containing 1 part of sodium pentacyanamino-ferronate and 5 parts lactose was devised for determining tuberculostatic drugs in urine. The method was sensitive to 10 µg/ml and took only 2-3 minutes. Cycloserine was indicate by a blue green color.

## 7. Pharmacokinetics

### 7.1 Absorption

Cycloserine is rapidly absorbed from the GIT. About 70-90% of an oral dose of cycloserine is absorbed from the GIT. Following a single 250-mg dose in healthy adults, peak plasma concentrations of drug averaging 10 µg/ml are attained within 3-4 hrs. In patients with normal renal function, some drug accumulation may occur in the first 3 days of cycloserine therapy. The best therapy results occur with trough serum concentrations of 25 to 30 µg/ml. Serum levels in excess of 30 µg/ml have been associated with toxicity and should be avoided (2, 42).

In an experimental study of the pharmacokinetics of cycloserine, isoniazid and p-aminosalicylic acid in rats with acute and chronic alcohol poisoning, Ortenberg (43) reported that the drugs were absorbed more slowly, reached lower blood concentrations and

were inactivated to a greater degree in rats with acute ethanol intoxication disturbed liver function and showed detoxification.

Zitkova and Tousek (44) presented a comparative study on the pharmacokinetics of cycloserine and terizidone carried out on 35 men aged 19-83 suffering from pulmonary tuberculosis. The patients are divided into three groups. One group received a single dose of 250 mg cycloserine, the second group 500 mg of each drug and the third 750 mg of each drug. Each patient serves as his own control. The patients in each group were subdivided into young and elderly patients. The results showed that terizidone blood concentrations were higher at all time intervals than the concentrations attained after the same doses of cycloserine, however, this increase was not proportional to two molecules of cycloserine contained in a molecule of terizidone. In elderly patients the blood concentrations of both drug were higher than in the younger patients.

The transport characteristics of cycloserine and its L-isomer were studied in everted sacs of rat colon in vitro and the buccal cavity of a female subject aged 25 years (45). The relationship between the rate of transport of the isomers against the initial concentration for both the rat colon and the human buccal mucosa indicated that the process involved was passive diffusion.

## 7.2 Distribution

Cycloserine is widely distributed into body tissues and fluids. In the lungs, ascitic fluid, pleural fluid and synovial fluid, the drug is distributed in concentrations approximately equal to plasma concentrations. CSF concentrations of cycloserine are reported to be 50-80% of concurrent plasma concentrations in patients suffering from inflamed meninges. The drug is not bound to plasma proteins. Cycloserine readily crosses the placenta and is distributed in milk (4). Nair et al. (46) reported that a substantial portion of the drug passed to the fetus and amniotic fluid before delivery. It is probable that the high concentration in the cord represents accumulation of the drug in this compartment. Also,

the infants' metabolizing enzyme system have still been too immature to adequately metabolize the antibiotic.

A recent review (47) of reports of antituberculosis drugs given during pregnancy shows information indicating placental transfer of many such drugs including cycloserine. For all these drugs the average maternal and fetal blood concentrations at birth are given.

Coletsos (48) in his study of the concentration of cycloserine in serum and viscera in the cynocephalic monkey, reported lung levels of cycloserine of 53  $\mu\text{g/g}$  (which is 5 times the in vitro bacteriostatic level) in 1 hr but then fell to levels below the bacteriostatic threshold after 10 hrs. In the kidney cycloserine accumulated more slowly but also left the tissue slowly. Neither the liver nor the spleen show significant tendency to accumulate cycloserine. In the chimpanzee as in the cynocephalic monkey, the rate at which cycloserine leaves the plasma differed from that at which it left the viscera.

### 7.3 Elimination

The plasma half-life of cycloserine is approximately 10 hrs., in patients with normal renal function. In patients with impaired renal function, plasma concentrations are higher and the half-life is prolonged. When an oral dose of cycloserine was given to patients with normal renal function, 60-70% of the dose was excreted unchanged in urine by glomerular filtration within 72 hrs. Small amounts of the drug were also excreted in feces. The remainder of the dose is thought to be biodegraded to unidentified metabolites (42).

The pharmacokinetics comparative study of Zitkova and Tousek (44) on cycloserine and terizidone has showed that the excreted quantity of terizidone in urine was higher, but the differences as compared with excreted cycloserine were not statistically significant. Patients with higher age average showed slower excretion rate in urine.

Coletsos (48) studied the elimination of cycloserine, given s.c. or in to guinea pigs, rabbits and chicken. The drug was almost completely eliminated in several

hours even at concentrations of 150 mg/kg. In monkeys, the drug excretion was delayed in direct relation to the distance in rank of the monkey from that of anthropoid monkeys. Elimination was rapid in the cynocephalic monkey, less so in the Papio sphinx and even slower and more prolonged (24 hrs.) in rhesus monkey. In the chimpanzee, cycloserine was slowly eliminated, hacillostatic level in the plasma being maintained for 24 hrs after parentral injection of 40 mg/kg.

#### Acknowledgement

The authors would like to thank Dr. R.R. Abou-Shaaban for performing the thermal analysis and X-ray powder diffraction experiments. The authors also wish to thank Mr. Tanvir A. Butt for typing the manuscript.

#### References

1. "The Merck Index", Tenth Edition, Merck & Co., Inc., Rahaway, New Jersey, 1983 p. 395.
2. "Index Nominum: International Drug Directory", Compiled by the Scientific Documentation Centre of the Swiss Pharmaceutical Society, Zurich, 1987.
3. "CRC, Atlas of Spectral Data and Physical Constants for Organic Compounds", Vol. III, Page 158, 1975. Edited by J.G. Grasselli and W.M. Ritchey, 2nd Edition, CRC Press Inc., Cleaveland, Ohio.
4. "AHFS, Drug Information 87", Edited by Gerald K. McEvoy, Published by Authority of the Board of Directors of the American Society of Hospital Pharmacists, 1987.
5. P.H. Hidy and E.B. Hodge, *J. Am. Chem. Soc.*, 77, 2345 (1955).
6. F.A. Kuehl, Jr., *J. Am. Chem. Soc.*, 77, 2344 (1955).
7. C. Stammer and J. McKinney, *J. Org. Chem.*, 30, 3466 (1965).

8. K. Fukushima and T. Arai, *Shitsuryo Bunseki*, 27(2), 107 (1979).
9. J.W. Lamb, in *Analytical Profile of Drug Substances*", Edited by K. Florey, Vol. 1, p. 53, Academic Press, Inc. 1972.
10. R.M. Evans, "The Chemistry of the Antibiotics Used in Medicine", p. 12, Pergamon Press, Oxford, New York, 1965.
11. R.L. Harned, P.H. Hidy and E.K. Baru, *Antibiotic and Chemotherapy*, 5, 204 (1955).
12. L.F. Yakhontova, B.P. Bruns, S.N. Kobzieva and N.A. Perevozskaya, *Antibiotiki*, 15, 411 (1970).
13. R.-Sheng Cai, S.-Fang Liang, S.-Li Yang, J.-Xin Wang and J.-Bao Zhong in *Nucleic Acids Proteins*, Proc. Symp.", 1979 (Pub. 1980), p. 581, Edited by Zhao-Wen Shen. Sci. Press: Peking, China.
14. M.L. Svensson and S. Gatenbeck, *Arch. Microbiol.*, 131(2), 129 (1982).
15. N. Isobe and S. Seki, *Iryo*, 22, 1201 (1968).
16. I. Cieslak, N. Buszko and Z. Makarowska-Plociennik, *Antibiotiki*, 12, 410 (1967).
17. T. Ohsato, *Kekkaku*, 45, 97 (1970).
18. V.D. Kartseva, B.P. Bruns, N.G. Kruzhkova and N.A. Bannova, *Khim.-Farm. Zh.*, 6, 54 (1972).
19. M. Medianu, A. Moldovan and A. Mataranga, *Revta Chem.*, 19(12), 734 (1968).
20. A. Braibanti, F. Dallavalle and G. Mori, *Ann. Chem.*, 71(5-6), 223-233 (1981).
21. V.D. Kartseva and B.P. Bruns, *Antibiotiki*, 10(4), 305-307 (1965).
22. Jones, *Analyt. Chem.*, 28, 39 (1956). Through Analytical Abstract, Vol. 13 No. 4394.

23. V.S. Svinchuk, V.F. Kramarenko and M.M. Orlinskii, *Probl. Tuberk*, 6, 64-66 (1979).
24. V.S. Svinchuk, V.P. Kramarenko and M.M. Orlinskii, *Farm. Zh.*, 1, 74-75 (1980).
25. V.S. Svinchuk, *Farm. Zh.*, 6, 46-49 (1982).
26. R.C. Hiremath and S.M. Mayanna, *Indian J. Pharm. Sci.*, 46, 19 (1984).
27. E. El-Sayed, Z.H. Mohamed and A.A.M. Wahbi, *Analyst*, 111, 915 (1986).
28. K.V. Uma, B. Jayaram and S.M. Mayanna, *Indian J. Pharm. Sci.*, 45, 170 (1983).
29. F. Saccani and G. Pitrolo, *Boll. Chim. Farm.*, 109, 388 (1970).
30. N.M. Lukovskaya and E.V. Mitropolitska, *Zh. analit Khim.*, 30(5), 985-998 (1975).
31. V.S. Svinchuk, *Farm. Zh.*, 6, 46-49 (1982).
32. M.E. Mohamed and M.S. Tawakkol, *J. Coll. Sci., King Saud Univer.*, 14(1), 113-118 (1983).
33. K.C. Guven and G. Ozsari, *Eczacilik. Bull*; 9(2), 19-29 (1967).
34. R. Voigt and A.G. Maa Bared, *J. Chromatogr.*, 36(1), 120-123 (1968).
35. C. Garber and J. Dobrecky, *Rev. Asoc. Bioquim. Argent.*, 33, 178-179 (1968).
36. L. David, F.E. Gainer and H.J. Wasselman, *J. Pharm. Sci.*, 62(8), 1344-1346 (1973).
37. D.G. Musson, S.M. Maglietto, S.S. Hwang, D. Gravellese and W.F. Bayne, *J. Chromatogr. Biomed. Appl.*, 414, 121 (1987).
38. H. Murakami, M. Kanzaki, C. Fujimoto and M. Haruta, *Shokuhin Eiseigaku Zasshi*, 12(2), 86-94 (1971).



39. L.M. Klyueva and N.I. Gelperin, *Khim. Farm. Zh.*, 9(6), 28-29 (1975).
40. United States Food and Drug Administration (Rockville, MD. 20857 U.S.A. Fed. Regist 30 Jun 1981 46 (125 Bk 1) 33512-13.
41. R.O. Shpits and M.D. Romanonko, *Proble. Tuberk.*, 3, 76-78 (1975).
42. "Drug Evaluations", Prepared by the American Medical Association, 6th Edition, 1986 p. 1539.
43. E.A. Ortenberg; *Probl. Tuberk.*, 9, 60 (1978).
44. L. Zitkova and J. Tousek, *Chemotherapy (Bassel)*, 20, 18 (1974).
45. S.A. Sprake and D.F. Evered; *J. Pharm. Pharmacol.*, 31, 113 (1979).
46. K.G.S. Nair, I.G. Epstein, H. Baron and M.G. Mulinos, *Antibiotics Annual*, 136-140, 1955-1956.
47. M.R. Holdiness, *Clin. Pharmacokinet.*, 13, 125 (1987).
48. P. Coletsos, *Scand. J. Resp. Dis. Suppl.*, 71, 40 (1970).

# **ANALYTICAL PROFILE OF 5-FLUOROURACIL**

**Said M. Bayomi and Abdullah A. Al-Badr**

**Department of Pharmaceutical Chemistry  
College of Pharmacy, King Saud University**

CONTENTS

1. Therapeutic function.
2. Description.
3. Physical Properties.
4. Spectral Properties.
  - 4.1 Ultraviolet Spectrum.
  - 4.2 Infrared Spectrum.
  - 4.3 Mass Spectra.
  - 4.4 Nuclear Magnetic Resonance Spectra.
  - 4.5 Thermal Analysis.
  - 4.6 X-Ray Powder Diffraction.
5. Chemical Properties.
6. Synthesis.
7. Metabolism.
8. Pharmacokinetics.
9. Clinical Toxicity.
10. Methods of Analysis.
  - 10.1 Identification.
  - 10.2 Fluorine Content.
  - 10.3 Spectrophotometric Analysis.
    - 10.3.1 Colorimetry.
    - 10.3.2 Ultraviolet Spectrometry.
    - 10.3.3 Infrared Spectrometry.
    - 10.3.4 Fluorine-19-NMR.
    - 10.3.5 Mass Spectrometry.
    - 10.3.6 Fluorometry.
  - 10.4 Chromatographic Analysis.
    - 10.4.1 Paper Chromatography.
    - 10.4.2 Thin-Layer Chromatography.
    - 10.4.3 High-Pressure Liquid Chromatography.

10.4.4 Gas Chromatography.

10.4.5 Gas Chromatography-Mass Spectrometry.

11. Electrochemistry.

Acknowledgement.

References.

## 5-FLUOROURACIL

### 1. THERAPEUTIC FUNCTION

5-Fluorouracil has been used in the treatment of cancer for more than two decades. It is a fluorinated antimetabolite of the pyrimidine uracil. It slows tumour cell growth by inhibiting thymidine formation, thereby inhibit protein synthesis by incorporating into RNA.

### 2. DESCRIPTION

#### 2.1 Nomenclature

##### 2.1.1 Chemical Names

5-Fluoro-2,4(1H,3H)-pyrimidinedione,  
2,4-Dioxo-5-fluoro pyrimidine,  
2,4(1H,3H)-pyrimidinedione, 5-fluoro.

##### 2.1.2 Generic Names

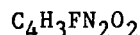
5-Fluorouracil, 5-FU, NSC 19893.

##### 2.1.3 Trade Names

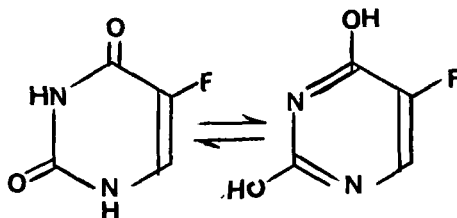
Adrucil, Arumel, Efudex, Efudix, Fluril, Fluracil, Fluoroplex, Fluoracil, Timazin.

#### 2.2 Formulae

##### 2.2.1 Empirical



##### 2.2.2 Structural



### 2.3 Molecular Weight

130.08

### 2.4 Research Number

Ro 2-9757

### 2.5 Chemical Abstracts Registry Number

[51-21-8]

### 2.6 Elemental Composition

C:36.93%, H:2.32%, F:14.61%, N:21.54%, O:24.60%.

### 2.7 Appearance, Color and Odor

White to practically white, odorless, crystalline powder (1), crystals from water or methanol (2).

## 3. PHYSICAL PROPERTIES

### 3.1 Melting Point

Melting point lies between 282 to 283°C with decomposition (3).

### 3.2 Thermodynamic and Physicochemical Parameters of 5-Fluorouracil (4)

The following thermodynamic and physicochemical parameters were predicted values based on the available heat of fusion, melting point, solubility parameters (of the drug and prospective solvents) and molar volume of 5-Fluorouracil (5-FU). Heat of fusion and melting point were determined by Du Pont (TA 9900) DSC unit (Fig. 7). The solubility parameter and the molar volume were calculated from structure using Fedor's (5) substituent constants. Heat of vaporization ( $\Delta H_v$ ), heat of mixing ( $\Delta H_m$ ), heat of dissolution ( $\Delta H_{diss}$ ), partition coefficients of 5-FU between different solvents, activity coefficient and solubility in different solvents were calculated using a developed (4) program which is based on the inter-related thermodynamic equations. These constants are listed in the following Table:-

Constants	Values	Constants	Values
Melting Point (deg. Kelvin)	551.85	Sol. in water (mol/L)	4.711537E-04
Heat of fusion (cal./mol)	8800	Sol. in glycerol (mol/L)	6.703796E-04
Heat of vaporization (cal/mol)	19694.43	Sol. in PG (mol/L)	1.109421E-04
Heat of sublimation (cal/mol)	28494.43	Sol. in methanol (mol/L)	8.535111E-05
Heat of mixing with water (cal/mol)	493.3872	Sol. in ethanol (mol/L)	1.856792E-05
Heat of mixing with octanol (cal/mol)	4569.321	Sol. in propanol (mol/L)	5.508214E-06
Heat of mixing with chloroform (cal/mol)	5543.161	Sol. in acetone (mol/L)	2.174898E-07
Heat of Dissolution in water (cal/mol)	9293.387	Sol. in dioxane (mol/L)	3.011725E-09
Heat of Dissolution in octanol (cal/mol)	13369.32	Sol. in n-hexane (mol/L)	2.176198E-09
Heat of Dissolution in chloroform (cal/mol)	14343.16	Sol. in chloroform (mol/L)	9.374298E-08
Molar volume (V)	47	Sol. in n-octanol (mol/L)	4.849687E-07
Log PC n-octanol	-2.987449		
Log PC chloroform	-3.701223		
Log Xi Ideal solubility	-2.96521		

### 3.3 Packing and Storage

Preserve in tight, light-resistant containers.

### 3.4 Caution

Great care should be taken to prevent inhaling particles of 5-fluorouracil and exposing the skin to it.

### 3.5 Loss on Drying

Dry it in vacuum over phosphorous pentoxide at 80° for 4 hours, it loses not more than 0.5% of its weight (3).

## 4. SPECTRAL PROPERTIES

### 4.1 Ultraviolet Spectrum

The ultraviolet spectrum of 5-fluorouracil in neutral methanol is depicted in Figure 1. The spectrum was recorded by DMS 90 spectrometer in the region of 200 to 400 nm.

The absorption maxima at 265 nm shifts to 210 nm due to Keto-enol tautomerization.

### 4.2 Infrared Spectrum

The infrared spectrum of 5-fluorouracil in a potassium bromide disc is presented in Figure 2. The spectrum was recorded with a Perkin-Elmer 1310 infrared spectrophotometer. The spectral assignments are presented in the following table:

<u>Frequency cm<sup>-1</sup></u>	<u>Assignment</u>
3122	NH Stretch
1718 and 1655	C = O, C = N- stretch
1425	
1243	CH in plane
812	CH out plane.

### 4.3 Mass Spectra

Figure 3 shows the 70 eV electron impact (EI) mass spectrum obtained on Varian MAT 311 mass spectrometer using ion source pressure of 10<sup>-6</sup> Torr, ion source temperature of 180°C and an emission current of 300 uA.



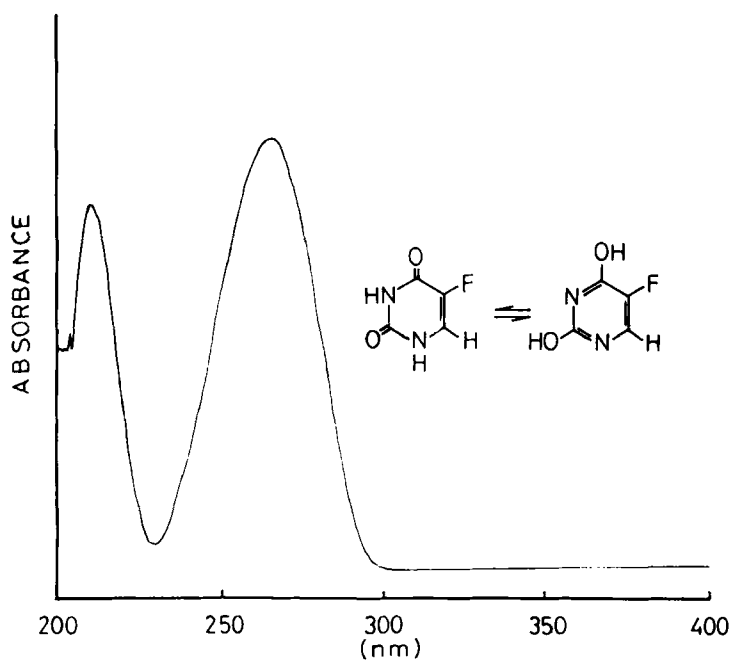


Figure 1: Ultraviolet spectrum absorption spectrum of 5-Fluorouracil in methanol.

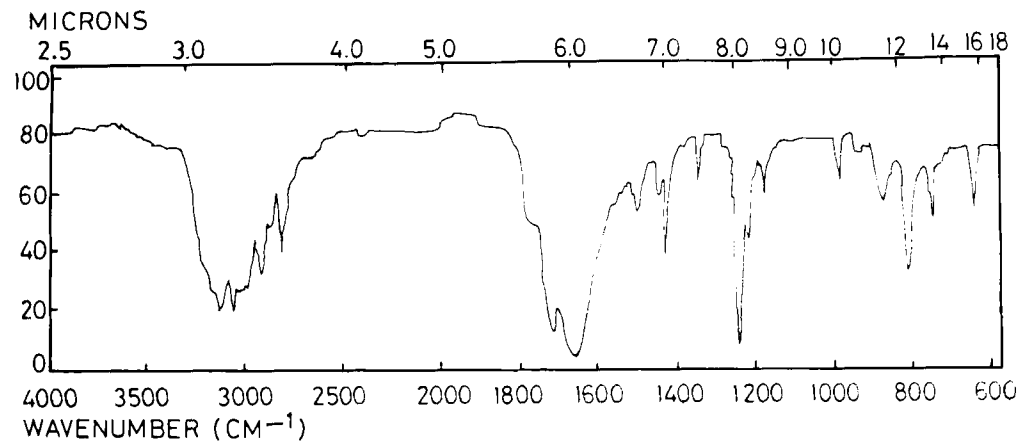


Figure 2: Infrared spectrum of 5-Fluorouracil (KBr disc).

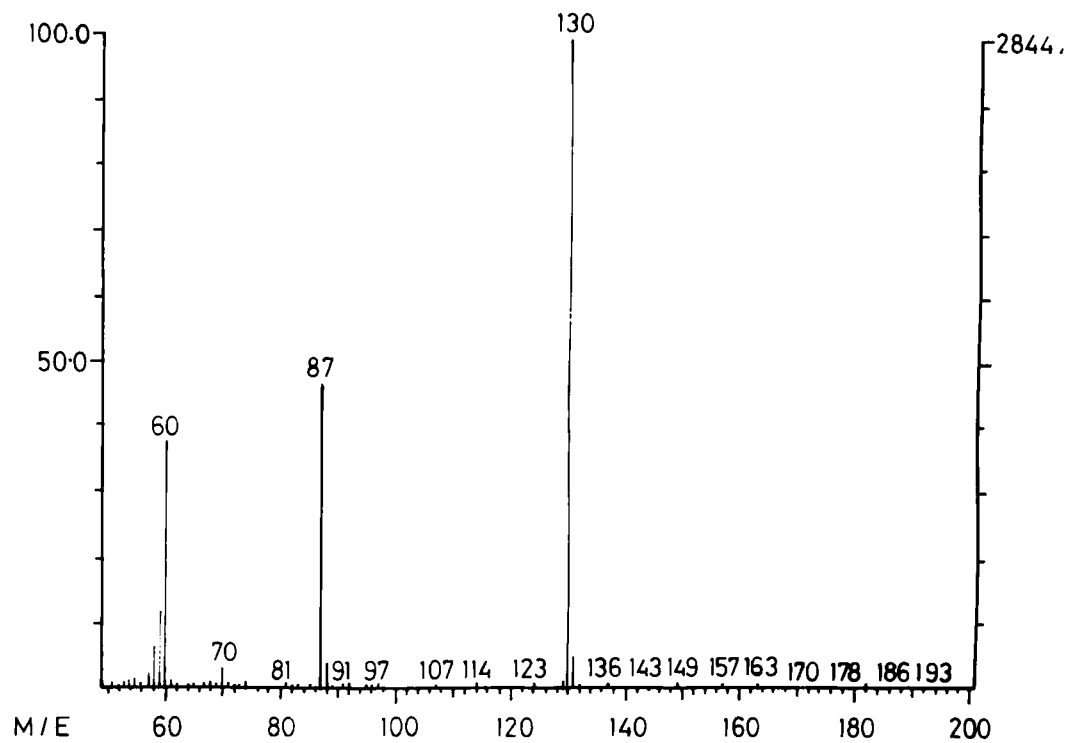


Figure 3: Electron Impact (EI) Mass spectrum of 5-Fluorouracil.

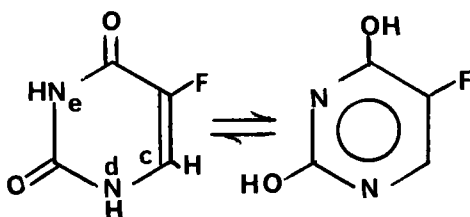
The spectrum is dominated by  $m/e$  87 ion (base peak) resulting from the loss of HNC $O$  from the parent and the fragment at  $m/e$  60 is formed by a hydrogen rearrangement giving half the ring with the fluorine substituent ( $C_2H_3FN$ ).

Chemical ionization (CI) spectrum is presented in Figure 4 and is obtained on a Finnigan 4000 Mass Spectrometer, with ion electron energy of 1000 eV, ion source pressure of 0.3 Torr, ion source temperature of 150 and emission current of 300  $\mu$ A. The mass spectral assignment of the only prominent ion under CI condition is 131 of  $M^+ + 1$ .

#### 4.4 Nuclear Magnetic Resonance Spectra (NMR)

##### 4.4.1 Proton NMR

The proton NMR spectrum, Figure 5, was recorded in dimethylsulfoxide- $d_6$  containing tetramethylsilane (TMS) as internal reference and with the use of a Varian XL 200 spectrometer. The spectral assignments are presented in the following Table:



<u>Proton assignment</u>	<u>Chemical shift (<math>\delta</math>) ppm</u>
a (DMSO- $d_6$ )	2.49
b (HDO)	3.43
c	7.80
d	10.80
e	11.45

##### 4.4.2 Carbon-13 NMR

The  $^{13}C$ -NMR spectrum shown in figure 6 was recorded

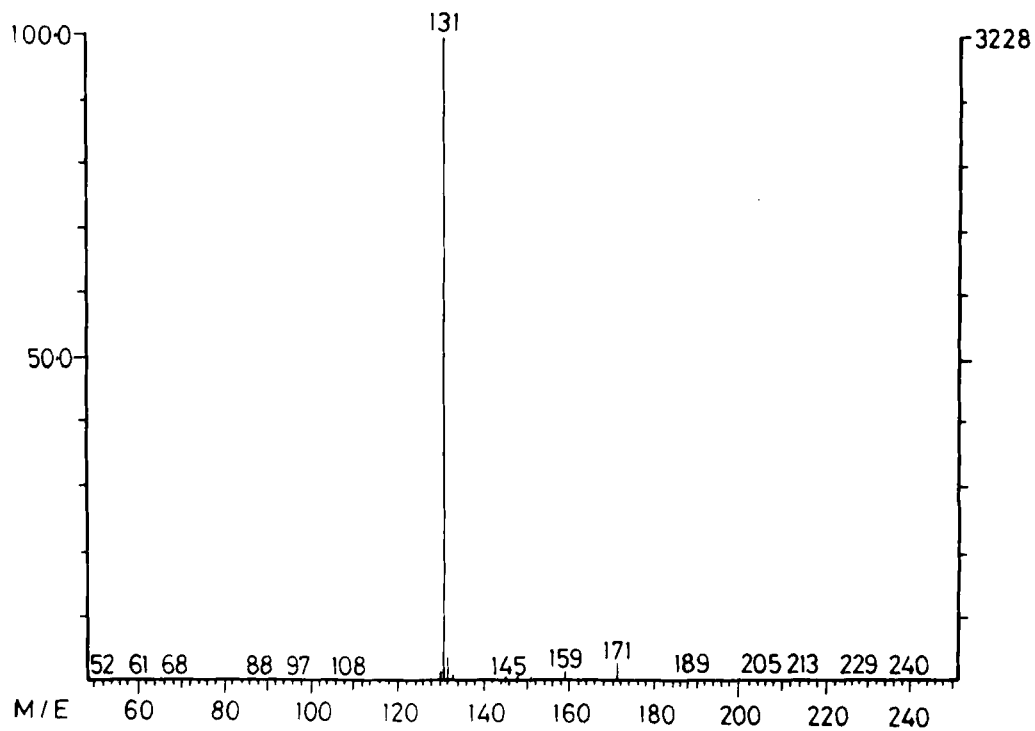


Figure 4: Chemical Ionization (CI) Mass spectrum of 5-Fluorouracil.

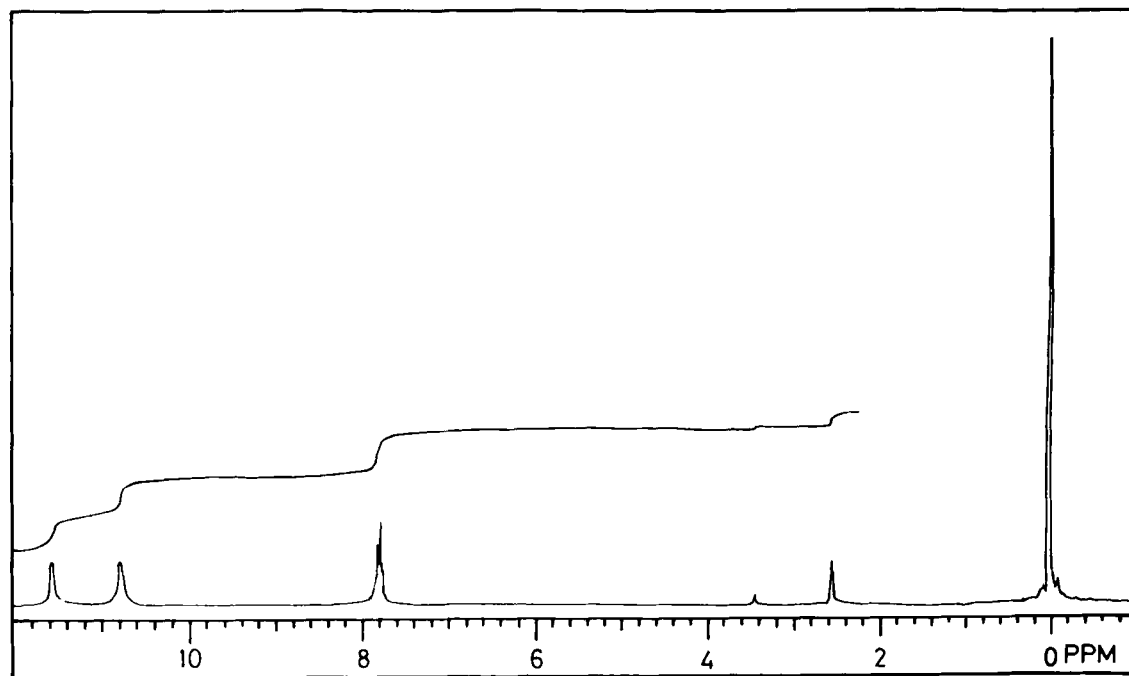


Figure 5: Proton NMR spectrum of 5-Fluorouracil in DMSO-d<sub>6</sub>.

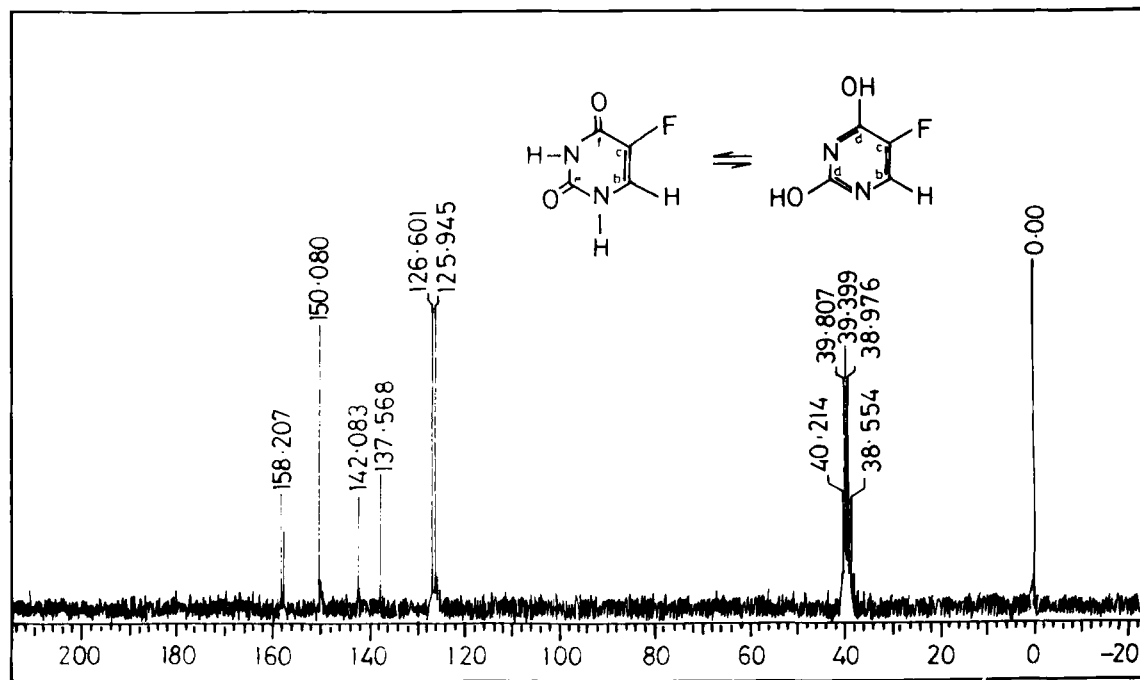
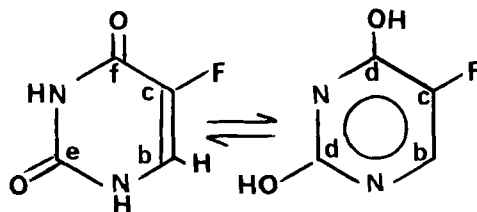


Figure 6:  $^{13}\text{C}$ -NMR Spectrum of 5-Fluorouracil in  $\text{DMSO}-d_6$ .

in deuterated dimethylsulfoxide using a Varian Associated Model XL-200 Spectrometer.

The spectral assignments are presented in the following Table:



<u>Carbon assignment</u>	<u>Chemical shift (<math>\delta</math>) ppm</u>
a (DMSO-d <sub>6</sub> )	39.4
b (Keto-enol)	{ 125.95 126.60
c (Keto-enol)	{ 137.57 142.08
d	150.08
e	158.00
f	158.21

#### 4.5 Thermal Analysis

The thermal analysis (Figure 7) was done on Du Pont (TA 9900) DSC unit between 160°C - 300°C at 10 C/min heating rate. The analysis of the data was done using purity program. The heat of fusion of 5-fluorouracil was found to be 8800 KCal/mole.

#### 4.6 X-Ray Powder Diffraction (4)

The X-ray diffraction pattern of 5-fluorouracil was determined using philips fully automated X-ray diffraction spectrogoniometer equipped with PW 1730/10 generator. Radiation was provided by a copper target (cu anode 2000 W,  $\lambda = 1.5480 \text{ \AA}$ ) high intensity X-ray tube operated at 40 KV and 35 MA. The monochromator was a curved single crystal one (PW1752/00). Divergence slit and the receiving slit were 1 and 0.1° respectively. The



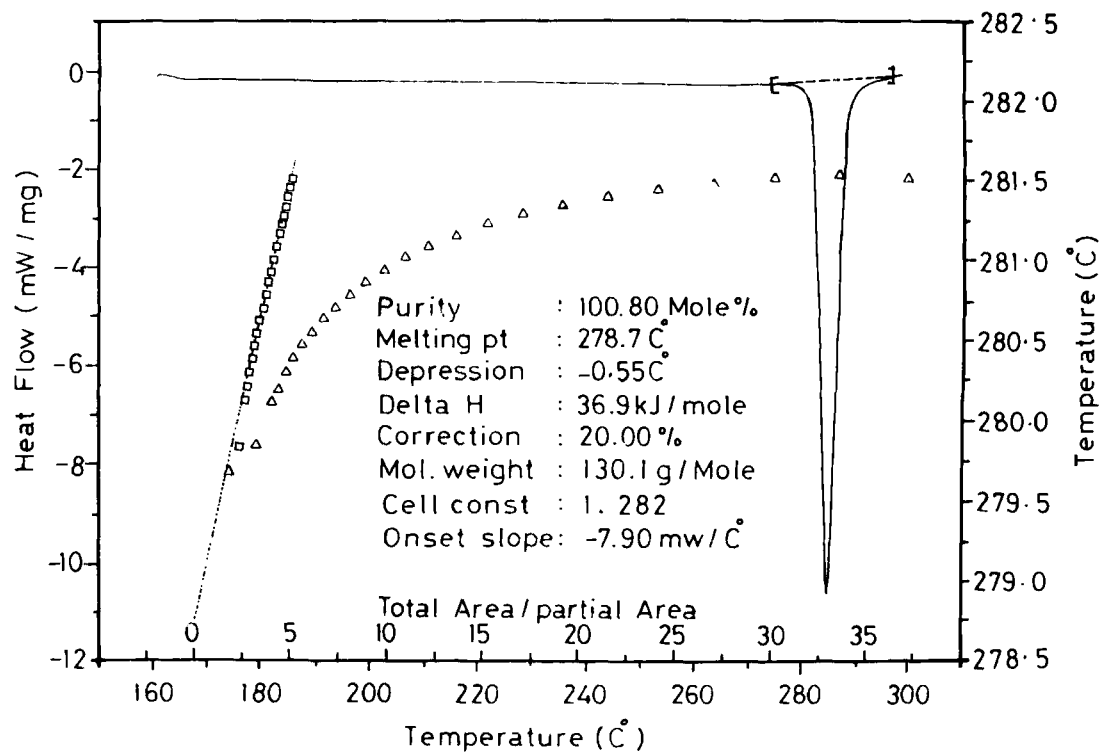


Figure 7: Thermal curve of 5-Fluorouracil.

scanning speed of the goniometer (PW1050/81) used was 0.022° per second. The instrument is combined with philips PM 8210 printing recorder with both analogue recorder and digital printer. The goniometer was aligned using silicon sample before use.

The X-ray pattern of 5-fluorouracil is presented in Figure 8. The interplanar distance and relative intensity are shown in the following table:-

X-Ray Diffraction Pattern of 5-Fluorouracil.

d(A)	I/I <sub>0</sub>	d(A)	I/I <sub>0</sub>
7.94	0.218	2.27	0.33
6.87	0.268	2.39	0.23
6.64	0.190	2.29	0.21
5.60	0.44	2.19	0.15
5.47	0.461	2.14	0.19
4.97	0.25	2.10	0.23
4.69	0.50	2.04	0.18
4.50	0.37	1.98	0.19
4.32	0.725	1.95	0.18
4.07	1.09	1.94	0.22
3.96	1.17	1.91	0.24
3.90	0.58	1.861	0.17
3.74	0.68	1.84	0.15
3.60	0.83	1.83	0.17
3.51	1.24	1.81	0.24
3.45	0.53	1.79	0.29
3.29	0.49	1.77	0.24
3.19	3.27	1.75	0.18
3.11	100	1.70	0.25
2.87	2.30	1.68	0.35
2.80	0.96	1.61	0.18
2.73	0.48	1.59	0.35
2.70	0.48	1.55	1.15
2.639	0.31	1.52	0.22
2.571	0.44	1.50	0.37
2.47	0.42	1.38	0.12

d = interplanar distance I/I<sub>0</sub> = relative intensity  
based on highest intensity of 100.

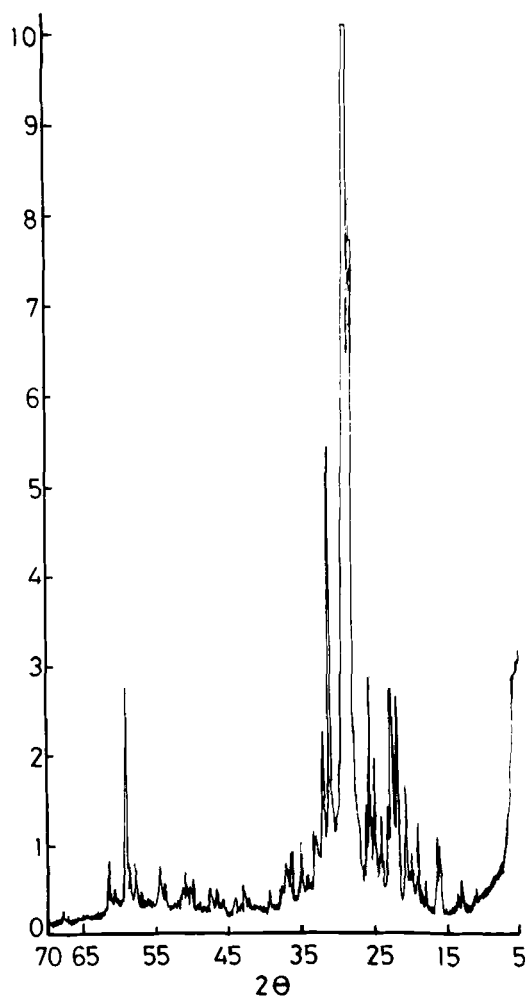
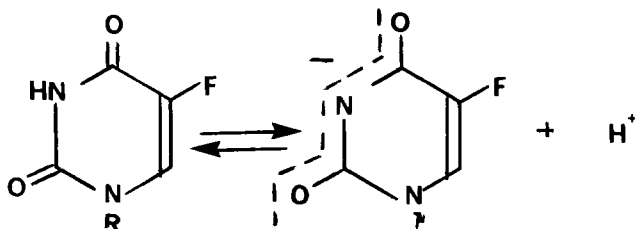


Figure 8: X-Ray powder diffraction pattern of 5-Fluorouracil.

## 5. CHEMICAL PROPERTIES

### 5.1 Effect of Flourine

Flourine has an inductive (electron withdrawing) effect, which is reflected in a much lower pKa with fluoro-uracil-containing compounds than with the natural compounds. The ionization that occurs is as follows:



In addition, the carbon-fluorine bond is stronger than the carbon hydrogen bond and is less susceptible to enzymatic cleavage. Thus substitution of a halogen atom of the correct dimension can produce a molecule that sufficiently resembles a natural pyrimidine to interact with enzymes of pyrimidine metabolism and also to interfere drastically with certain other aspects of pyrimidine action (6).

### 5.2 Hydrolysis

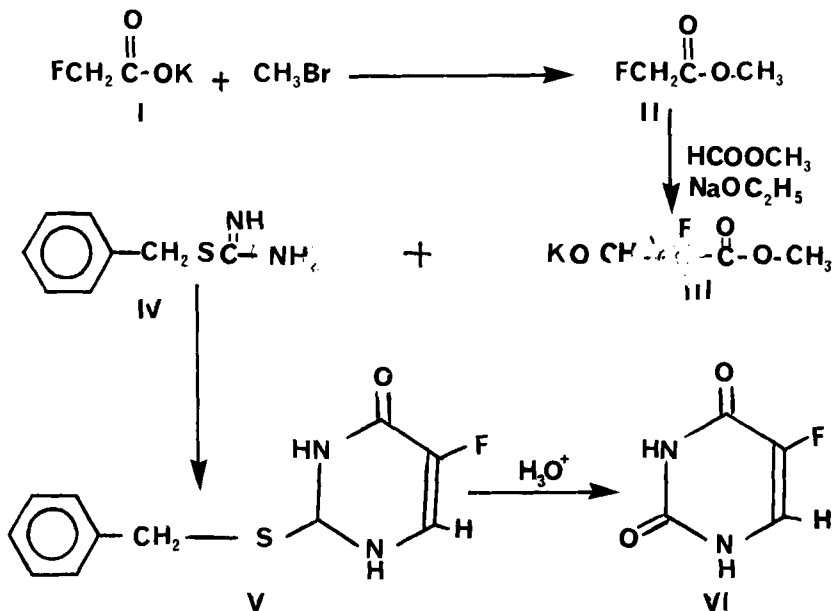
5-fluorouracil is stable in solutions up to pH 9. When 5-FU subjected to strongly basic conditions, it is hydrolyzed to urea, fluoride, and an aldehyde. This hydrolysis is enhanced by increased pH and temperature. Some of the urea formed on hydrolysis reacts further giving ammonia and CO<sub>2</sub> (1).

## 6. SYNTHESIS

a) Potassium fluoroacetate (I) is reacted with methyl bromide to form methyl fluoroacetate (II) which is then subjected to Claisen condensation with methyl formate and sodium ethoxide to produce the potassium enolate of the methyl ester of fluoromalonaldehyde (III). Cyclization of III is effected through condensation under anhydrous conditions with S-benzylisothiourea

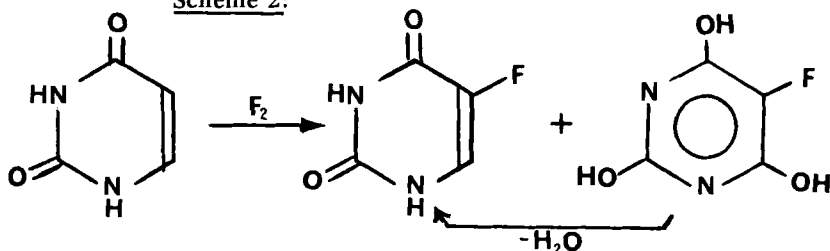
(IV). The resulting 2-(benzylthio)compound(V) is readily hydrolyzed in the presence of acid to 5-fluorouracil (VI), Scheme 1 (7).

Scheme 1:



b) Bubbling fluorine through an aqueous solution of uracil at 50-90° gave 5-fluorouracil and 5-fluoro-5,6-dihydro-2,4,6-trihydropyrimidine which yields 5-fluorouracil on dehydration by refluxing an aqueous solution. Optimum yields, 26-78%, of 5-fluorouracil were obtained with a  $\text{F}_2/\text{N}_2$  vol. ratio  $\approx 2:1$ , Scheme 2 (8).

Scheme 2:



c) 5-Fluorouracil was prepared by the procedure described in Scheme 1 using ethylfluoroacetate as starting material and 2-ethyl-2-thiopseudourea hydrobromide instead of benzylisothiurea (1).

## 7. METABOLISM

5-Fluorouracil is metabolized extensively in the liver and its concentration decline rapidly to undetectable level within 2 hours. As plasma 5-fluorouracil concentration decline, concentrations of its major metabolites, 5,6-dihydro-5-fluorouracil (fluorouracil-H2),  $\alpha$ -fluoro- $\beta$ -ureido-propionic acid (FUPA) and  $\alpha$ -fluoro- $\beta$ -guanido-propionic acid (FABL) increase (9). Fluorouracil-H2 is detectable within 5-minutes of administration of 5-fluorouracil with peak plasma concentrations of 23.7  $\mu\text{mol/L}$  occurring after 1 hour (10). It was reported that fluorouracil-H2 is an important active fluoropyrimidine catabolite (11).

The liver converts fluorouracil-H2 to fluorouracil-PA and FBAL by a dose-dependent saturable system. Fluorouracil-PA and FBAL peak serum concentrations are detectable approximately 90 minutes after infusion (12).

Inactivation of 5-fluorouracil by the liver during continuous regional and systemic infusion in pigs was reported by Almersjo et al (13).

Fluorouracil is converted intracellularly to 5-fluoro-2'-deoxyuridylylate (FdUMP) by a series of enzymatic reactions. Initially, 5'-monophosphate nucleotide (FUMP) is formed either by orotate phosphoribosyl transferase in the presence of 5-phosphoribosyl-1-pyrophosphate (PRPP), or by the action of uridine phosphorylase and then uridine kinase (14) (Figure 9). The FUMP is further metabolized to diphosphate (FUDP) and then to triphosphate (FUTP) which can be incorporated into RNA thus producing a fraudulent RNA. However, the primary activation steps of fluorouracil involves the formation of the deoxymonophosphate (FdUMP) by the reduction with ribonucleotide reductase to FUDP and then by the action of the phosphorylase to FdUMP.

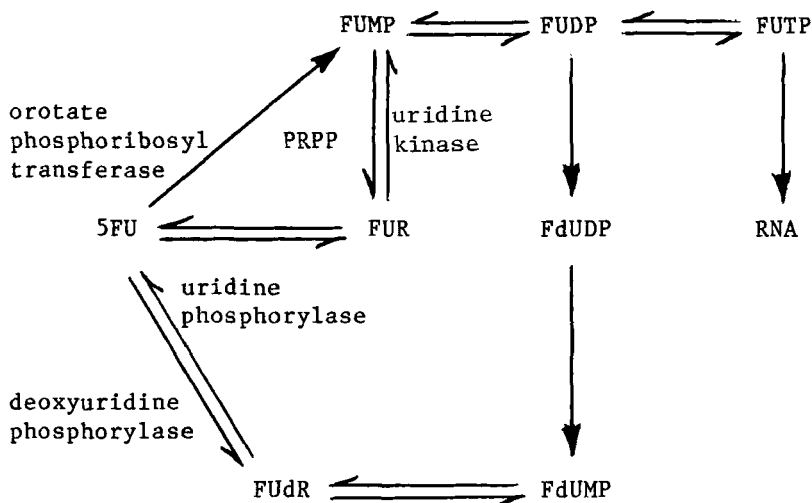


Figure 9: Intracellular activation pathways of fluorouracil (14).

Metabolism of 5-fluorouracil in sensitive and resistant Novikoff hepatoma (15) and tumor cells (16) was reported.

When 1-(2-tetrahydrofuryl)-5-fluorouracil added to an incubation medium containing human maxillary cancer cells was converted to 5-fluorouracil dose-and time-dependently (17).

Experimental study of an increase in fluorouracil activity under the influence of phenobarbital, glutathione, and uracil was reported (18,19).

Comparison of 5-fluorouracil metabolism in two human gastrointestinal tumor cell lines was reported (20).

Studies on the ability of thymidine to modify the chemotherapeutic activity and metabolism of 5-fluorouracil and 1- $\beta$ -D-arabinofuranosylcytosine in rat and mice was reported by Danhauser (21).

## 8. PHARMACOKINETICS

### 8.1 Absorption

5-Fluorouracil is most commonly administered

intravenously. Oral preparations as tablets, syrups and solutions has been used, although in most cases absorption is unpredictable by this route. Responses after oral dosing appear to be shorter and fewer when compared with intravenous dosing (22).

After oral doses of 5-fluorouracil 15 to 20 mg/kg/day, bioavailability may range from 50 to 80% (23). Absorption can be increased by buffering the solution with 0.2 mol/L bicarbonate buffer (pH=9) on with administration of 100 ml of water. Acidic fruit juices recommended in the past may actually precipitate the drug.

After a 200 mg oral dose of 5-fluorouracil peak serum concentrations may range from 0.5 to 1.0 mg/L within 15 to 30 minutes, depending on the oral preparation used, and the presence or absence of food in the stomach (24).

## 8.2 Distribution

5-fluorouracil rapidly distributes to most tissues with a  $V_d$  of  $8.84 \pm 3.90 \text{ L/m}^2$ . AUC is reported to be greater than  $7125 \pm 237/\mu \text{ mole/L. min.}$  following an intravenous dose of  $500 \text{ mg/m}^2$  (10). After a 15 mg/kg intravenous bolus dose, 5-fluorouracil penetrates the CSF producing peak concentrations of 60 to 80 nmol/L. Although penetration across the placental barrier has not been investigated in humans, 5-fluorouracil is known to be a teratogen in animals and is assumed to cross the placenta.

## 8.3 Elimination

Urinary excretion of intravenously injected 5-fluorouracil- $2\text{-}^{14}\text{C}$ , given as a single dose, amounts to only 11% in 24 hours; however, during this period, 63% of the radioactivity is expired as carbon dioxide. Given by continuous intravenous infusion for 24 hours, plasma concentration in the range of 0.5 to  $3.0 \mu\text{M}$  are obtained and the urinary excretion of 5-fluorouracil is only 4%, while the  $^{14}\text{CO}_2$  excretion rises to 90% (25).

5-Fluorouracil-H<sub>2</sub> represents 1% of total metabolites eliminated by the kidney, while FBAL accounts for more than 70%. Only minor amounts of 5-fluorouracil and fluorouracil-PA are detected in the urine.



Pharmacokinetics studies, orally administered ftorafur was absorbed well along with food, and its bioavailability was similar to that observed after i.v., administration approximately the same amounts of 5-fluorouracil were formed after oral and i.v. administration, and oral administration of ftorafur was a safer way to deliver 5-fluorouracil to tumor cells than was the oral administration of 5-fluorouracil itself (26).

#### 9. CLINICAL TOXICITY (6)

The clinical manifestations of toxicity caused by fluorouracil and floxuridine are similar and may be difficult to anticipate because of their delayed appearance. The earliest untoward symptoms during a course of therapy are anorexia and nausea; these are followed shortly after by stomatitis and diarrhea, which constitute reliable warning signs that a sufficient dose has been administered. Stomatitis is manifested by formation of a white patchy membrane that ulcerates and becomes necrotic. The occurrence of similar lesions in the stoma of colostomies and at post-mortem examination of the gastrointestinal tract, as well as complaints of dysphagia, retrosternal burning, and proctitis, indicates that enteric injury may occur at any level. The major toxic effects, however, result from the myelosuppressive action of these drugs; clinically, the effects are most frequently manifested as leukopenia, the nadir of which is usually between the ninth and fourteenth day after the first injection of drug. Thrombocytopenia and anemia may complicate the picture. Loss of hair, occasionally progressing to total alopecia, nail changes, dermatitis, and increased pigmentation and atrophy of the skin may be encountered. Neurological manifestations, including an acute cerebellar syndrome, have been reported, and myelopathy has been observed after the intrathecal administration of fluorouracil. The low therapeutic indices of these agents emphasize the need for very skillful supervision by physicians familiar with the action of the fluorinated pyrimidines and the possible hazards of chemotherapy (6).

## 10. METHODS OF ANALYSIS

### 10.1 Identification

The United States Pharmacopeia XXI (1985) (3) describes the following identification tests:

**A:** The infrared absorption spectrum of a mineral oil dispersion of it exhibits maxima only at the same wavelengths as that of a similar preparation of USP Fluorouracil RS.

**B:** The ultraviolet absorption spectrum of a 1 in 100,000 solution in a pH 4.7 acetate buffer (prepared from 8.4 g of sodium acetate and 3.35 mL of glacial acetic acid mixed with water to make 1000mL) exhibits maxima and minima at the same wavelengths as that of a similar solution of USP Fluorouracil RS, concomitantly measured, and the respective absorptivities, calculated on the dried basis, at the wavelength of maximum absorbance at about 266 nm do not differ by more than 3%.

**C:** To 5 mL of a solution (1 in 100) and 1 mL of bromine water TS: the bromine color is discharged.

### 10.2 Fluorine Content

US Pharmacopeia 1985 (3) describes the assay of fluorine 5-fluorouracil as follows:

**Fluorine content**--[Note - All laboratory utensils used in this procedure should be scrupulously clean and free from even trace amounts of fluoride. The use of plasticware, wherever possible, in the preparation and storage of solutions and for measurement of potentials is recommended].

Isopropyl alcohol solution - Dilute 295mL of isopropyl alcohol with water to 500 mL.

Buffer solution - To 55 g of sodium chloride in a 1-liter volumetric flask add 500 mg of sodium citrate, 255 g of sodium acetate and 300 mL of water. Shake to dissolve, and add 115 mL of glacial acetic acid. Cool to room temperature, add 30 mL of isopropyl alcohol, dilute with water to volume, and mix. The pH of the resulting solution is between 5.0 and 5.5.

Reagent blank - Pipet 15 mL of 1,2-dimethoxyethane into a flat-bottom, glass-joint, 500-mL flask, and proceed as directed under Test preparation, beginning with "add the contents of a 15-mL vial of sodium biphenyl solution."

Modified calomel reference electrode - Mix 70 mL of a freshly prepared saturated potassium chloride solution with 30 mL of isopropyl alcohol, fill the electrode with the clear supernatant liquid, and allow the electrode to soak in the remainder of the solution for a minimum of 2 hours before using. Store the electrode immersed in the potassium chloride-isopropyl alcohol solution when not in use.

Standard stock solution - Weigh accurately 2.211 g of sodium fluoride, previously dried at 150° for 4 hours, into a 1-liter volumetric flask, and dissolve in about 200 mL of water. Add 1 mL of sodium hydroxide solution (1 in 25), dilute with water to volume, and mix. Store this solution in plastic containers. One mL is equivalent to 1 mg of fluoride.

Standard curve - Dilute 10.0 mL of Standard Stock solution with water to 100 mL. Into each of four 100-mL volumetric flasks pipet 0.8, 1.0, 1.2 and 1.6 mL, respectively, of the resulting solution. To each flask add 15 mL of Reagent blank, dilute with Buffer solution to volume, and mix. Use these dilutions, containing, respectively, 0.8, 1.0, 1.2 and 1.6  $\mu\text{g}$  per mL, to construct the standard curve as follows. Determine the potentials of each solution as directed under Procedure. Plot the results of fluorine concentration as the abscissa, in mg per 100 mL versus the potential, as the ordinate, on semilogarithmic graph paper, for each of the standards. Draw the best straight line through the plotted points.

Test preparation - Place 200 mg of Fluorouracil, accurately weighed, in a 250-mL volumetric flask, add about 150 mL of 1,2-dimethoxyethane, shake by mechanical means to dissolve, dilute with the same solvent to volume, and mix. Pipet 15 mL of this solution into a flat-bottom, glass-joint, 500-mL flask, add the contents of a 15-mL vial of sodium biphenyl solution through a long-stem funnel to prevent splattering, swirl the flask gently, and cover with a watch crystal. Allow to stand

at room temperature for 20 minutes, then cautiously add 50.00 mL of isopropylalcohol while swirling the flask. Add 10.0 mL of 30 percent hydrogen peroxide and 4.0 mL of 1 N sodium hydroxide, and connect the flask to a water-cooled reflux condenser that previously has been cleaned with water and isopropyl alcohol and dried. Place the flask on a hot plate, set at about  $245^{\circ}$ , and reflux for 1 hour. Cool to temperature, rinse the condenser with 15mL of Isopropyl alcohol solution, transfer the contents of the flask to a 250-mL volumetric flask using Isopropyl alcohol solution as a rinse, dilute with the same solvent to volume, and mix. Pipet 15 mL of this solution into a 100 mL volumetric flask, and dilute with Buffer solution to volume.

Procedure - Measure the potential, in mV, of the Test preparation with a suitable pH meter having a minimum reproducibility of  $\pm 0.2$  mV, and equipped with a fluoride-specific ion electrode and a glass-sleeved Modified calomel reference electrode. When taking a measurement, immerse the electrodes into the solution, which has been transferred to a 150-mL plastic beaker, insert a suitable plastic-coated stirring bar, place the beaker on a magnetic stirrer, taking adequate precautions to prevent heat transfer, and stir for 2 minutes before reading. Dry the electrodes between measurements, taking care not to scratch the crystal surface of the specific ion electrode. Determine the quantity of fluorine, in mg per 100 mL of Test preparation, from the Standard curve. Multiply the quantity by the factor 138.9 to express the result as percentage. Not less than 13.9% and not more than 15.0% of fluorine, calculated on the dried basis, is found.

Assay - 0.1 N Tetrabutylammonium hydroxide in methanol - Dilute with methanol a commercially available solution of tetrabutylammonium hydroxide in methanol, and standardize as directed under Tetrabutylammonium Hydroxide, Tenth-Normal (0.1 N).

Procedure - Transfer about 400 mg of Fluorouracil, accurately weigh to a 250-mL conical flask, add 80 mL of dimethylformide and warm gently to dissolve. Cool, add 5 drops of 1 in 100 solution of thymol blue in dimethylformamide, and titrate with 0.1 N Tetrabutylammonium hydroxide in methanol to a blue end-point, taking precautions to prevent absorption of atmospheric

carbon dioxide. Perform a blank determination, and make any necessary correction. Each mL of 0.1 N tetrabutylammonium hydroxide is equivalent to 13.01 mg of  $C_4H_3FN_2O_2$ .

### 10.3 Spectrophotometric Analysis

#### 10.3.1 Colorimetry

Hassib (27) reported a qualitative and quantitative analysis of two uracil anticancer drugs including 5-fluorouracil. The drugs were selectively identified and estimated in pure and in dosage forms by means of color reactions. 5-Fluorouracil was treated with bromine water in borox medium, and then with 2,4-dinitrophenylhydrazine in acidic medium to give an orange-red precipitate which produces a distinctly violet solution when treated with potassium hydroxide solution. From 100 gm sample containing 30 ug of 5-fluorouracil, 29  $\mu$ g were recovered by this method.

Li (28) reported the separation of 5-fluorouracil from 5-fluorocytosine. The mixture was stirred in 10% hydrochloric acid at  $5-10^\circ$  for one hour to precipitate 5-fluorouracil. The filtrate was made alkaline with 20%  $NH_4OH$  to precipitate 5-fluorocytosine. The latter was decolorized by active carbon and washed with acetone to give 5-fluorocytosine with 99% purity.

#### 10.3.2 Ultraviolet Spectrometry

Gaussian analysis of absorption spectra for a 0.01 N sodium hydroxide solution containing 5-fluorouracil at 270 and 300 nm have been reported by Tikhvinskaya and Egerts (29). It showed the molar extinction coefficients to be 4570 and 3000 respectively. Determination of the difference in extinction coefficients for the drug is suggested for quantitative analysis of the drug in the presence of the others.

Borodavkin et al (30) have studied the absorption ultraviolet spectroscopy and electronic structure of some 5-substituted analogs of pyrimidines.

### 10.3.3 Infrared Spectrometry

The vibrational spectra of 5-fluorouracil and of 6-azauracil have been reported by Rai (31). The IR absorption spectra and intensities of 5-fluorouracil (42 frequencies) were recorded at 200-4000  $\text{cm}^{-1}$ . Tautomeric behavior of the molecules was noted; these molecules were ketonic. The fundamental (24-frequencies) of uracil, 6 azauracil and 5-fluorouracil were correlated.

### 10.3.4 Fluorine-19-Nuclear Magnetic Resonance

Marshall and Smith (32) have introduced, a fluorine containing nuclear spin-label, at uracil residues of 5 S RNA from *E. coli*. The 19-F-NMR spectrum of the labeled RNA showed widely dispersed signals with short relaxation times, indicating  $\geq 4$  distinct uracil environment and a relatively rigid solution structure. On heating to 72°, the  $^{19}\text{F}$  NMR spectrum collapses to a single dominant signal, corresponding to a common chemical environment for virtually all the exposed uracil residues. Denaturation was reversible as was judged by appearance of the  $^{19}\text{F}$ -NMR spectra before and after heating. Advantages of the F nuclear spin-label compared to prior  $^1\text{H}$ ,  $^{13}\text{C}$ ,  $^{15}\text{N}$  and  $^{31}\text{P}$  NMR studies of RNA were discussed.

Burnell *et al* (33) have determined the complete fluorine chemical shift tensor from the moment of the magnetic resonance lineshape. The  $^{19}\text{F}$  chemical shift tensors were reported for fluoranil and for 5-fluorouracil using the magnetic field dependence of the second and third moments of their fluorine magnetic resonance spectra. The values obtained at 25° for the principal chemical shift tensor components and the asymmetry parameters are 55 ppm and 0.3 for 5-fluorouracil.

### 10.3.5 Mass Spectrometry

Marunaka (34) reported the electron impact mass spectra for 5-fluorouracil and some N-substituted derivatives. Characteristic fragment ions were produced by retro-Diel-Alder decomposition of the fluorouracil skeleton.

Marunaka et al (35) have also reported the field desorption, chemical ionization and electron-impact mass spectra of 5-fluorouracil and derivatives. The mass spectra contained characteristic patterns formed by retro-Diel-Adler reaction which were useful for structural identification.

#### 10.3.6 Fluorimetry

Bueyvektimkin (36) assayed 5-fluorouracil fluorometrically after derivatization with dansyl chloride followed by ion-pair extraction. The reaction between the drug and the fluorescent reagent was optimum at pH 10 after 45 minutes yielding 3-N-dansyl-5-fluorouracil which was separated by thin-layer chromatography on silica gel G plates. The plates were developed in chloroform-methanol (9:1). Excitation was at 366 nm and emission was measured at 520 nm. This procedure was utilized for measuring concentrations of added 5-fluorouracil in 0.25-1 ml samples of cat plasma with a detection range of 40-400 ng. The method was also applied to measuring known quantities of the drug in pharmaceutical preparation.

### 10.4 Chromatographic Analysis

#### 10.4.1 Paper Chromatography

The drug was separated by descending chromatography using whatman 3 mm paper with isopropanol-conc. ammonia-water (7:1:2) or 0.5 mM  $\text{Na}_3\text{B}_4\text{O}_7$ -isopropanol (1:2) as the solvent system. The compound was also separated by electrophoresis on a Savant flat-plate high-voltage instrument at 1000 V for 60 minutes in 0.1 M hydrochloric acid-potassium chloride buffer (pH 1.8). The spots were visualized with UV light (254 nm) in both systems. The electrophoretic mobilities of the drug was 5.9 cm, from the original towards the anode (37).

#### 10.4.2 Thin-Layer Chromatography

Thin-Layer chromatography-densitometric assay of 5-fluorouracil in blood plasma and in pharmaceutical preparations was presented by Bueyuektimkin (38). The drug is extracted using ion-pair extraction with

tetrabutylammonium hydrogen phosphate and cellulose with methylene dichloride as the eluting material. After evaporation to dryness, the residue was dissolved in methanol and the solution spotted on ready for use silica gel plates, developed with ethyl acetate, and after evaporation of the solvent, the plates were scanned in 266 UV light. The drug was also determined by treating the plates with Gibbs reagent solution and scanning the purple spots at 660 nm. Using UV detection, a linear relation exists between 100-500 ng of the drug and peak height. Using visible densimetry, a linear relation exists between 50-600 ng of the drug and peak heights. Both methods appear to be convenient for routine analysis.

Thin-layer chromatography was used for simulations determination of some radiosensitizing and chemotherapeutic drugs in plasma (39) the samples are heated with borate buffer and then extracted on a Sep-Pak C<sub>18</sub> cartridge of subjected to solvent extraction with isopropanol-ethyl acetate (1:4) followed by centrifugation. Thin-layer chromatography was performed on silica gel G-60 F<sub>254</sub> plates and the spots were quantitated by scanning densitometry. The Sep-Pak extraction did not give good result and the solvent extraction procedure was effective for 5-fluorouracil.

#### 10.4.3 High-Pressure Liquid Chromatography (HPLC)

Several HPLC methods have been reported in the literature for the quantitative determination of 5-fluorouracil and of its metabolites in biological fluids.

Rustum (40) described a high-pressure liquid column chromatography method for the separation and identification of ribonucleosides and deoxyribonucleosides and bases. The separation was carried out with different pH values and buffers, namely, phosphate buffer containing 2.5% methanol at pH 6.9 and 3 or 50 mM Na<sub>3</sub>BO<sub>3</sub> buffer, pH 9.0. These different conditions were utilized to obtain more definitive identification and quantitation of normal metabolites and their antimetabolites. The method



described the quantitation of plasma metabolites and antimetabolites and of fluoropyrimidine.

Mori et al (41) have investigated 1-hexylcarbamoyl-5-fluorouracil and its metabolites in human body fluids by high-pressure liquid chromatography and gas liquid chromatography with election capture detection (ECD-GLC). The compound was extracted with chloroform from serum sample was determined by HPLC. 5-Fluorouracil separated from the compound and its oxidative metabolites using Amerlite XAD-2 resin was determined by ECD-GLC after chloromethyldimethylsilylation. The compound and its metabolite having 5-fluorouracil nucleus were converted into 5-fluorouracil under alkaline conditions and then determined by ECD-GLC and this expressed as total 5-fluorouracil. The detection limit of the drug was 10 ng/ml.

Complete analysis of the metabolites of 5-fluorouracil in cell extracts have been reported by Pogolotti et al (42). A high-pressure liquid chromatography system for separation of the metabolites of the drug found in acid-soluble cell extracts is described. An example of the integrated methodology in which all metabolites of 5-fluorouracil are analyzed after 6 hour exposure of L1210 cells to (6-<sup>3</sup>H) fluorouracil provided.

Katsumata (43) has reported a method for the analysis of contents of 5-fluorouracil in tissues by high-performance chromatography. Rabbit and human tissues of lip, gingiva or buccal mucosa were homogenized with water, mixed with 0.1 volume of 60% perchloric acid, dentrifuged at 12000 r.p.m. (10 minutes). The supernatant was mixed with an aqueous volume of ethyl acetate and the aqueous layer was analyzed for 5-florouracil by HPLC using a liquid chromatography column (ISC-07/S1504) and an eluant of 0.02 M NaH<sub>2</sub>PO<sub>4</sub> at 140-180 kg/cm<sup>2</sup>. Serum was similarly treated with perchloric acid and ethyl acetate successively.

#### 10.4.4 Gas Chromatography

Christophidis et al (44) have described a comparison of liquid and gas-liquid chromatographic assay of 5-

fluorouracil in plasma. Advantages of liquid over gas-chromatography procedure are the simpler extraction procedure, elimination of the need for a derivatization step with silylating agents, and a 20-fold greater sensitivity, the minimum detectable concentration of the drug in plasma is 25 ug/l. The enhanced sensitivity enabled measurement of the concentrations of the drug found in plasma of patients receiving continuous intravenous infusions of the drug; such concentrations are generally unmeasurable by gas-chromatographic methods. Liquid and gas chromatographic measurement on 36 plasma samples obtained from patients after rapid I.V. injection of the drug were compared.

Driessen et al (45) reported a gas liquid chromatographic assay of 5-fluorouracil in blood plasma. Gas chrom Q was used as the support material, 3% Versamid 930 was the absorbent, and 5 chlorouracil the internal standard. A sensitivity of 1 ng/injection was reported.

An improved gas-liquid chromatographic assay of the drug in plasma was described by Van den Berg et al (46) using the chloromethyl derivative of the drug and a  $^{63}\text{Ni}$  electron-capture detector. The column was packed with chromosorb W HP coated with 3% OV-1 and the carrier gas was nitrogen with a flow rate of 40 ml/minutes. The column, injector, and detector temperature were 230, 230 and 280° respectively, the drug was extracted for plasma with propanol (16%) solution. After evaporation of the organic phase to dryness the residue was derivatized in ethyl acetate.

Gelijkens et al (47) have described the preparation and capillary gas chromatographic properties of volatile derivatives of 18 pyrimidine and purine nucleic acid bases (N,O-peralkyl and trifluoroacetyl-N,O-alkyl derivatives).

De Bruijn et al (48) reported a gas chromatographic assay for the determination of the drug and 5,6-dihydrofluorouracil. The selectivity and sensitivity of the method allows the determination of both compounds in 200 ul of plasma. Diphenylsuccinimide and chlorouracil were used as external and internal

standard respectively. The drug and its dihydro derivative plasma concentration of a number of patients with breast cancer treated with the drug were determined in order to demonstrate the usefulness of this method.

De Bruijn et al (49) have also determined the underivatized antineoplastic drugs including 5-fluorouracil and some of their metabolites by capillary gas chromatography combined with electron-capture and nitrogen-phosphorous selective detection. The method is rapid and sensitive for the determination of plasma 5-fluorouracil and some of its metabolites in one analysis. Surface-coated open-tubular OV-375 column was used, with electron-capture detection and nitrogen-phosphorous-selective detection.

#### 10.4.5 Gas Chromatography-Mass Spectrometry

Several authors have reported the use of gas chromatography-mass spectrometry methods for the determination of 5-fluorouracil in biological fluids.

A method was described (50) for determining 5-fluorouracil in plasma using methylated thymine as an internal standard. The drug was extracted from plasma by a novel procedure which removed plasma component interfering with the sensitivity of the assay. The method included heating the plasma, washing with ether and extraction of the drug under optimum conditions. The sensitivity of the assay was 10 mg of 5-fluorouracil/ml plasma sufficient to determine the low concentration of the drug found in plasma during continuous infusion of the drug in patients receiving chemotherapy for cancer.

Sadee et al (51) have described a method for the determine 5-fluorouracil in blood plasma, which involves extraction with ammonium sulphate and water-preanol (4: 1 v/v), formation of the ditrimethylsilyl derivative, and gas chromatography-mass fragmentation analysis. The method detect 1 ng/ml in most, but not all samples.

Min and Garland (52) have developed a rapid gas chromatographic-chemical ionization mass spectro-

metry (GC-CIMS) procedure to measure 5-fluorouracil in human plasma. An analog of the drug was added to plasma as the internal standard. Plasma was treated with an equal volume of saturated ammonium sulphate solution, washed with benzene and extracted with 20% propanol in ether. The organic phase was evaporated and the residue was methylated with ethereal diazomethane. A portion of the solution obtained after removal of the derivatizing agent and reconstitution of the residue in acetone was injected into a 4 ft by 2 mm glass column packed with 3% POLY 1-110 on 100-120 mesh GCQ. Isobutane was used both as the gc carrier and as the ci reagent gas. The mass spectrometer was set to monitor m/e 159 (MH of the drug) and m/e 161 (MH<sup>+</sup> of <sup>15</sup>N<sub>2</sub> of the drug) in the gc effluent. Standard curve, obtained by analysing plasma samples spiked with known amounts of the drug, were used to convert the ion ratio of m/e 159 to m/e 161 in an unknown sample of the concentration of the drug.

5-fluorouracil was detected as a metabolite during a quantitative determination of 1,3-bis (tetrahydro-2-furanyl)-5-fluoro-3,4-pyrimidinone and its metabolites in plasma by high-pressure liquid chromatography and gas chromatography-mass fragmentography (53). After acidification, the plasma was extracted with chloroform. The remaining aqueous layer containing the drug was neutralized and extracted with ethyl acetate, and further purified, the thymine internal standard added and the mixture was silylated. An aliquot of the drug was chromatographed on a column packed with 3% OV-17 on Chromosorbs WAW and their subjected to mass fragmentograph.

Lakings et al (54) have analysed 5-fluorouracil quantitatively in human serum by selected ion monitoring gas chromatography-mass spectrometry. Plasma samples containing the drug were prepared by ultra filtration and separation of the drug on an AG 1-X2 anion-exchange resin column in the acetate form (after elution with 0.1 N acetic acid). The drug was redissolved in methylene chloride and silylated with a BSTFA-CH<sub>3</sub>CN mixture. The compound was detected on a gas chromatography-mass spectrometer with a column packed with 3% Dexsil 300 on 100-200 mesh supelcoport at an initial temperature of 100°

increasing to  $250^{\circ}$  at  $16^{\circ}/\text{min}$ . Helium was the carrier gas. The mass spectrometer was operated at 70 eV with a source temperature of  $240^{\circ}$  and the dodecapole at  $110^{\circ}$ . The  $m/e$  ion 259.1 was selected for quantitation of the drug. The method permits detection of the drug at sufficiently low level to enable pharmacokinetic analysis.

A method was described by Jsomura et al (55) as simple, sensitive and specific for determining 5-fluorouracil in plasma after administration of 1-hexylcarbamoyl-5-fluorouracil, using gas-chromatography-mass spectrometry. Thymine was used as an internal standard. After removal of interfering substances with chloroform, ether, and Amberlite XAD-2- resin. The drug and thymine were extracted with 16% propanol in ether and methylated with trimethyl anilinium hydroxide. Forgment ion at  $m/e$  158 and 154, the molecular ion of the dimethyl derivative of the drug and thymine, respectively, were used to monitor the drug and thymine.

Comparative determination of 5-fluorouracil in plasma using gas chromatography/mass spectrometry and high-pressure liquid chromatography was reported by Aubert et al (56). The procedures have been developed to determine the drug in human plasma. The internal standard was 5-bromouracil. For HPLC assay used an extracted plasma with an ether-isopropanol mixture (75:25), the analysis were performed on a u Porssil column (10 m) with hexane ethanol (75:25) as an eluent solvent. For GC/MS after precipitation of protein, the plasma was purified through a micro column packed with AGI X 4 resin and then the component were methylated. Electron impact and selective ion monitoring modes were retained for GC/MS quantitative studies. The two techniques were compared by measurement on plasma samples obtained from patients after rapid iv injection, infusions and oral administration.

Mori et al (57) determined metabolites in tissues after administration of 1-hexylcarbamoyl-5-fluorouracil. After oral administration of the compound to rats, the concentration of 5-fluorouracil and its analogs in liver were determined simply and accurately by gas-chromatography-mass spectrometry.

Concentrations of the drug as low as 0.01 ug/g were determined. The use of 5-chlorouracil as an interval standard increased the accuracy of the determination. The time of the determination was only minutes/sample.

#### 11. ELECTROCHEMISTRY

Palecek et al (58) have studied the reaction of purine and of pyrimidine derivatives with the electrode mercury. The electrochemical behaviour of 30 purine and pyrimidine derivatives and of a further 12 derivatives containing sulfur or halogen (e.g., 5-fluorouracil) were studied. Interaction of pyrimidine derivatives with mercury (II) in homogenous aqueous solutions were investigated. A correlation was found between the ability of these substances react with mercury in solution and from precipitates, and to yield anodic polarographic current.

#### 12. ISOTACHOPHORESIS

Analytical isotachophoresis for the determination of 5-fluorouracil in plasma, was described by Gustavsson et al (59). The inclusion of spacers in the system greatly improved the separation and quantitation. The method can be employed for simultaneous measurement of different fluorinated pyrimidines used in clinical practice.

#### ACKNOWLEDGEMENT

The authors wish to thank Mr. Altaf H. Naqvi, for typing the manuscript.

#### REFERENCES

1. B.C. Rudy, B.Z. Senkowski, in Analytical Profiles of Drug Substances Vol. 2, K. Florey, Ed. (Academic Press, New York, (1973) pp. 221.
2. Merck Index 10th Ed., M. Windholz (Editor) page 4092, Merck and Co. Inc. Rahway, N.J. 1983.
3. United States Pharmacopeia, XXI, 440 (1985).
4. Abou-Shaaban R.R.A. Personal Communication.

5. Fedors R.F. Polymer Eng. Sci., 14, 147-154 (1974).
6. Goodman and Gilman's, The Pharmacological Basis of Therapeutics, 7th ed. Macmillan Publishing Co., Inc New York, 1267 (1985).
7. U.S. Pat. 2, 802, 005.
8. P.D. Schuman, P. Tarrant, D.A. Warner, Westmoreland, Geraldine (PCR Inc.) Can. 985, 681 (Cl. 260-258), 16 Mar (1976); US Appl. 658, 645, 07 Aug. 1967; Ch. Abst. Vol. 85, 1976, 46746.
9. J.M. Kirkwood, W. Ensminger, and A. Rosowsky. N. Papathanasopoulos. E. Frei. Cancer Research 40, 107 (1980).
10. G.D. Heggie, J.P. Sommadossi, D.S. Cross, W.J. Hasler and R.B. Diasio, Cancer Research, 47, 2203 (1987).
11. R.B. Diasio, J.D. Schuetz, H.J. Wallace and J.P. Sommadossi, Cancer Research 45, 4900 (1985).
12. E.A. dr Bruijin, L. Remyer, U.R. Tjaden, C. Erkelens and L.M. de Brauw, et al. Biochemical Pharmacology 35 (15), 2461 (1986).
13. O. Almersjo, A. Brandberg, B. Gustavsson, L.O. Halstrons and T. Seeman. Eur. Surg. Res. 8(6) 489 (1976).
14. C. Heidelberger., in Handbook of Experimental Pharmacology, Vol. 38 Part II Sartorelli, A.C. and Zohns. D, Gi., Eds., Springer-Verlog, New York. 193 (1975). From CRC Series in Medical Chemistry Chemotherapeutic Agents Vol. II, 262 (1986) CRC Press, Inc. Boca Rator, Florida.
15. S.D. Wilkinson, and A.J. Gumley, J. Biol. Chem. 252 (3), 1051 (1977).
16. M. Fukushima, K. Ikenaka, T. Shirasaka and S. Fujii, Gann. 70(1), 47 (1979).
17. T. Yoneda, T. Ogawa and M. Sakuda, Gan to Kagaku Ryoho., 10(4) 1207 (1983) (Japan).
18. H. Fujita, EKsp. Klin. Farmakoter, 12, 91 (1983).

19. T. Taguchi, EKsp. Klin. Farmakoter, 12, 205 (1983).
20. W.L. Washtien, Cancer Res. 44(3), 909 (1984).
21. L.L. Danhauser, From Diss. Abstr. Int. B. 42(9) 3647 (1982).
22. G.D. Hahn, C.G. Moertel, A.J. Schutt and H.W. Bruckner. Cancer 35, 1031 (1975).
23. S.A. Jacobs, R.G. Stoller, B.A. Chabner and D.G. Johns, J. Clin. Inv., 57, 534 (1976).
24. M. Kurihara, K. Miyasaka, T. Izumi, Y. Sasaki and T. Kamano. In Kimura et al. (Eds) Fluoropyrimidines in cancer therapy, pp. 229-241, Tokyo (1984).
25. K.L. Mukherjee and C.J. Heidelberg. Bio. Chem. 235, 433 (1960).
26. R.B. Diasio, H.L. Hunter, J.A. LaBudde, R.F. Mayol and H.P. Browder, EKsp. Klin. Farmakoter. 12, 31 (1983).
27. S.T. Hassib, Talanta, 28 (9), 685 (1981).
28. N. Li, Yiyao Gongye 7, 36 (1983).
29. T.I. Tikhvinskaya and V. Egerts., Nauchn. Konf. Khim. Anal. Pribalt. Resp. B. SSR (Tezisy Dokl) 1st, 47 (1974).
30. A.V. Borodavkin, E.I. Budovskii, Y.S. Dolin, Y.V. Morozov, F.A. Savin, V.O. Chekhov and Y.Y. Yakovlev, Usp. Kvantovoi Khim. Kvantovoi Biol., Tr. Mezhdunar Konf., 1, 45 (1980).
31. J.N. Rai, Indian J. Phys., 57B (3), 241 (1983)
32. A.G. Marshall and J.L. Smith, J. Am. Chem. Soc., 99(2) 635 (1977).
33. E.E. Burnell, A.L. MacKay, D.C. Roe and A.G. Marshall, J. Magn. Reson., 45 (2) 344 (1981).
34. T. Marunaka, Biomed. Mass. Spectrum., 8 (3), 105 (1981).
35. T. Marunaka, Y. Umeno, Y. Minami and T. Shibata, Biomed. Mass Spectrum., 7 (8), 331 (1980).



36. N. Bueyuektimkin, Sci. Pharm., 51(1), 26 (1983).
37. R.B. Silverman and L.V. Kapili, J. Chromatogr. 161, 415 (1978).
38. N. Bueyuektimkin, Sci. Pharm. 51 (1), 21 (1983).
39. D. Tonelli, E. Gattavecchia and A. Breccia, J. Chromatogr., 275 (1), 223 (1983).
40. Y.M. Rustum, Anal. Biochem., 90 (1), 289 (1978).
41. K. Mori, H. Kobe, H. Namekawa, H. Misono, Y. Yokomama and T. Kobari. Chromatography (Tokyo), 29 (3) 314 (1981).
42. A.L. Pogolotti Jr., P.A. Nolan and D.V. Santi, Anal. Biochem., 117 (1) 178 (1981).
43. I. Katsumata, Shigaku, 70 (5), 869 (1983).
44. N. Christophidis, G. Mihaly, F. Vajda and W. Louis, Clin. Chem., 25 (1), 83 (1979).
45. O.M.J. Driessen, P.J.A. Timmermans and D. DeVos, Appl. Methods Oncol., 1, 149 (1978).
46. H.W. Van der Berg, R.F. Murphy, R. Hunter and D.T. Elmore, J. Chromatogr., 145 (2), 311 (1978).
47. C.F. Gelijkens, D.L. Smith and J.A. McCloskey, J. Chromatogr., 225 (2), 291 (1981).
48. E.A. De Bruijn, O. Driessen, N. Van den Bosch, E. Van Strijen, P.H.T.J. Snee, A.T. Van Oosterom and U.R. Tjaden, J. Chromatogr., 278 (2) 283 (1983).
49. E.A. De Bruijn, U.R. Tjaden, A.T. Van Oosterom, P. Leeftang and P.A. Leclercq, J. Chromatogr., 279 603 (1983).
50. B.L. Hillcoat, M. Kawai, P.B. McCulloch, J. Rosenfeld and C.K.O. Williams, Br. J. Clin. Pharmacol., 3 (1), 135 (1976).
51. W.Sadee, C. Finn and J. Staroscik, Adv. Mass Spectrum.

- Biochem. Med., 1, 509 (1976).
52. B.H. Min and W.A. Garland, Res. Commun. Chem. Pathol. Pharmacol., 22(1) 145 (1978).
53. T. Marunaka and Y. Umeno, J. Chromatogr., 157 (1), 321 (1978).
54. D.B. Lakings, R.H. Adamson and R.B. Diasio, J. Chromatogr., 146 (3), 512 (1978).
55. H. Isomura, S. Higushi and S. Kawamura, J. Chromatogr., 224 (3) 423 (1981).
56. C. Aubert, C. Luccioni, P. Coassolo, J.P. Sommodossi and J.P. Cano, Arzneim-Forsch., 31 (12), 2048 (1981).
57. K. Mori, M. Fuse and T. Kohari, Yakuri to Chiryo, 11(11), 4655 (1983).
58. E. Palecek, F. Jelen, H. Mac Anh and J. Lasovsky, Bioelectrochem. Bioenerg. 8 (6) 621 (1981).
59. B. Gustovsson, O. Almersjoe, M. Berne and J. Waldenstroem, J. Chromatogr., 276 (2) 395 (1983).

## ERRATA

### SPIRONOLACTONE—Volume 4, p. 431

It has been pointed out by J. McB. Miller of the European Pharmacopoeial Commission that the infra-red spectrum of Spironolactone (Fig. 1), presented on page 434 of Volume 4, exhibits an intense absorption band at about  $770\text{ cm}^{-1}$ , indicative of the presence of residual chloroform in the preparation.

Miller has provided a KBr (chloroform free) spectrum of Spironolactone, representing the European Pharmacopoeia Reference Substance (Fig. 1).

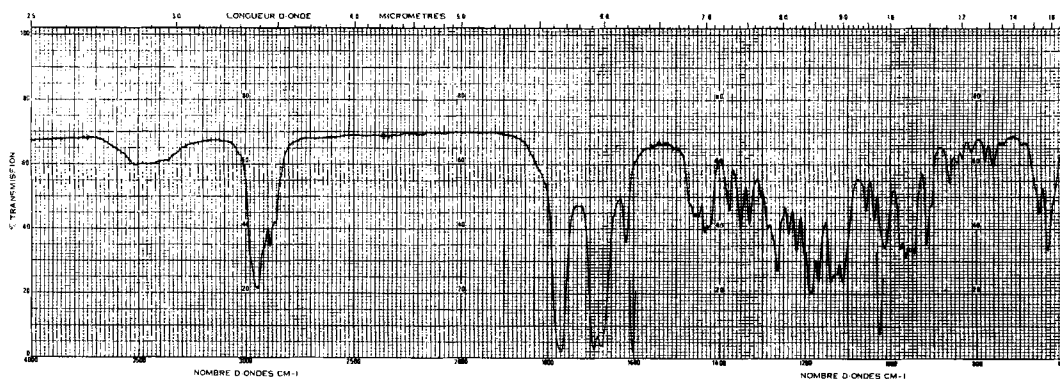


Figure 1. Infrared Spectrum of Spironolactone - KBr disc.

## CUMULATIVE INDEX

Bold numerals refer to volume numbers

- Acetaminophen, **3**, 1; **14**, 551  
 Acetohexamide, **1**, 1; **2**, 573  
 Allopurinol, **7**, 1  
 Alpha-tocopheryl acetate, **3**, 111  
 Amantadine, **12**, 1  
 Amikacin sulfate, **12**, 37  
 Amiloride hydrochloride, **15**, 1  
 Aminoglutethimide, **15**, 35  
 Aminophylline, **11**, 1  
 Aminosalicylic acid, **10**, 1  
 Amitriptyline hydrochloride, **3**, 127  
 Amoxicillin, **7**, 19  
 Amphotericin B, **6**, 1; **7**, 502  
 Ampicillin, **2**, 1; **4**, 518  
 Ascorbic acid, **11**, 45  
 Aspirin, **8**, 1  
 Atenolol, **13**, 1  
 Atropine, **14**, 32  
 Azathioprine, **10**, 29  
 Azintamide, **18**, 1  
 Aztreonam, **17**, 1  
 Bacitracin, **9**, 1  
 Baclofen, **14**, 527  
 Bendroflumethiazide, **5**, 1; **6**, 597  
 Benperidol, **14**, 245  
 Benzocaine, **12**, 73  
 Benzyl benzoate, **10**, 55  
 Betamethasone dipropionate, **6**, 43  
 Bretylium tosylate, **9**, 71  
 Bromazepam, **16**, 1  
 Bromocriptine methanesulfonate, **8**, 47  
 Busulphan, **16**, 53  
 Caffeine, **15**, 71  
 Calcitriol, **8**, 83  
 Camphor, **13**, 27  
 Captopril, **11**, 79  
 Carbamazepine, **9**, 87  
 Cefaclor, **9**, 107  
 Cefamandole nafate, **9**, 125; **10**, 729  
 Cefazolin, **4**, 1  
 Cefotaxime, **11**, 139  
 Cefoxitin, sodium, **11**, 169  
 Cephalixin, **4**, 21  
 Cephalothin sodium, **1**, 319  
 Cephradine, **5**, 21  
 Chloral hydrate, **2**, 85  
 Chlorambucil, **16**, 85  
 Chloramphenicol, **4**, 47, 518; **15**, 701  
 Chlordiazepoxide, **1**, 15  
 Chlordiazepoxide hydrochloride, **1**, 39; **4**, 518  
 Chloroquine, **13**, 95  
 Chloroquine phosphate, **5**, 61  
 Chlorothiazide, **18**, 33  
 Chloropheniramine maleate, **7**, 43  
 Chlorprothixene, **2**, 63  
 Chlortetracycline hydrochloride, **8**, 101  
 Chlorthalidone, **14**, 1  
 Chlorzoxazone, **16**, 119  
 Cholecalciferol, *see* Vitamin D<sub>3</sub>  
 Cimetidine, **13**, 127; **17**, 797  
 Cisplatin, **14**, 77; **15**, 796  
 Clidinium bromide, **2**, 145  
 Clindamycin hydrochloride, **10**, 75  
 Clioquinol, **18**, 57  
 Clofazamine, **18**, 91  
 Clofibrate, **11**, 197  
 Clonazepam, **6**, 61  
 Clorazepate dipotassium, **4**, 91  
 Clotrimazole, **11**, 225  
 Cloxacillin sodium, **4**, 113  
 Cocaine hydrochloride, **15**, 151  
 Codeine phosphate, **10**, 93  
 Colchicine, **10**, 139  
 Cyanocobalamin, **10**, 183  
 Cyclizine, **6**, 83; **7**, 502  
 Cyclobenzaprine hydrochloride, **17**, 41  
 Cycloserine, **1**, 53; **18**, 567  
 Cyclosporine, **16**, 145

Cyclothiazide, **1**, 66  
 Cyproheptadine, **9**, 155  
 Dapsone, **5**, 87  
 Dexamethasone, **2**, 163; **4**, 519  
 Diatrizoic acid, **4**, 137; **5**, 556  
 Diazepam, **1**, 79; **4**, 518  
 Dibenzepin hydrochloride, **9**, 181  
 Dibucaine and dibucaine hydrochloride, **12**, 105  
 Diflunisal, **14**, 491  
 Digitoxin, **3**, 149  
 Digoxin, **9**, 207  
 Dihydroergotoxine methanesulfonate, **7**, 81  
 Dioctyl sodium sulfosuccinate, **2**, 199; **12**, 713  
 Dipiperodon, **6**, 99  
 Diphenhydramine hydrochloride, **3**, 173  
 Diphenoxylate hydrochloride, **7**, 149  
 Disopyramide phosphate, **13**, 183  
 Disulfiram, **4**, 168  
 Dobutamine hydrochloride, **8**, 139  
 Dopamine hydrochloride, **11**, 257  
 Doxorubicine, **9**, 245  
 Droperidol, **7**, 171  
 Echothiophate iodide, **3**, 233  
 Emetine hydrochloride, **10**, 289  
 Enalapril maleate, **16**, 207  
 Ephedrine hydrochloride, **15**, 233  
 Epinephrine, **7**, 193  
 Ergonovine maleate, **11**, 273  
 Ergotamine tartrate, **6**, 113  
 Erythromycin, **8**, 159  
 Erythromycin estolate, **1**, 101; **2**, 573  
 Estradiol, **15**, 283  
 Estradiol valerate, **4**, 192  
 Estrone, **12**, 135  
 Ethambutol hydrochloride, **7**, 231  
 Ethynodiol diacetate, **3**, 253  
 Etomidate, **12**, 191  
 Etoposide, **18**, 121  
 Fenopropfen calcium, **6**, 161  
 Flucytosine, **5**, 115  
 Fludrocortisone acetate, **3**, 281  
 Flufenamic acid, **11**, 313  
 Fluorouracil, **2**, 221; **18**, 599  
 Fluoxymesterone, **7**, 251  
 Fluphenazine decanote, **9**, 275; **10**, 730  
 Fluphenazine enanthate, **2**, 245; **4**, 524  
 Fluphenazine hydrochloride, **2**, 263; **4**, 519  
 Flurazepam hydrochloride, **3**, 307  
 Furosemide, **18**, 153  
 Gentamicin sulfate, **9**, 295; **10**, 731  
 Glibenclamide, **10**, 337  
 Gluthethimide, **5**, 139  
 Gramicidin, **8**, 179  
 Griseofulvin, **8**, 219; **9**, 583  
 Guanabenz acetate, **15**, 319  
 Halcinonide, **8**, 251  
 Haloperidol, **9**, 341  
 Halothane, **1**, 119; **2**, 573; **14**, 597  
 Heparin sodium, **12**, 215  
 Heroin, **10**, 357  
 Hexestrol, **11**, 347  
 Hexetidine, **7**, 277  
 Homatropine hydrobromide, **16**, 245  
 Hydralazine hydrochloride, **8**, 283  
 Hydrochlorothiazide, **10**, 405  
 Hydrocortisone, **12**, 277  
 Hydroflumethiazide, **7**, 297  
 Hydroxyprogesterone caproate, **4**, 209  
 Hydroxyzine dihydrochloride, **7**, 319  
 Impenem, **17**, 73  
 Imipramine hydrochloride, **14**, 37  
 Indomethacin, **13**, 211  
 Iodamide, **15**, 337  
 Iodipamide, **2**, 333  
 Iopamidol, **17**, 115  
 Iopanoic acid, **14**, 181  
 Isocarboxazid, **2**, 295  
 Isoniazide, **6**, 183  
 Isopropamide, **2**, 315; **12**, 721  
 Isoproterenol, **14**, 391  
 Isosorbide dinitrate, **4**, 225; **5**, 556  
 Ivermectin, **17**, 155  
 Kanamycin sulfate, **6**, 259  
 Ketamine, **6**, 297  
 Ketoprofen, **10**, 443  
 Ketotifen, **13**, 239  
 Khellin, **9**, 371  
 Leucovorin calcium, **8**, 315  
 Levallorphan tartrate, **2**, 339  
 Levarterenol bitartrate, **1**, 49; **2**, 573; **11**, 555  
 Levodopa, **5**, 189  
 Levothyroxine sodium, **5**, 225  
 Lidocaine base and hydrochloride, **14**, 207; **15**, 761  
 Lithium carbonate, **15**, 367  
 Lorazepam, **9**, 397  
 Maprotiline hydrochloride, **15**, 393  
 Mebendazole, **16**, 291  
 Mefloquine hydrochloride, **14**, 157  
 Melfhalan, **13**, 265  
 Meperidine hydrochloride, **1**, 175  
 Meprobamate, **1**, 209; **4**, 520; **11**, 587  
 6-Mercaptopurine, **7**, 343

Mestranol, **11**, 375  
 Methadone hydrochloride, **3**, 365; **4**, 520; **9**, 601  
 Methaqualone, **4**, 245, 520  
 Methimazole, **8**, 351  
 Methotrexate, **5**, 283  
 Methoxsalen, **9**, 427  
 Methyclothiazide, **5**, 307  
 Methylphenidate hydrochloride, **10**, 473  
 Methypylon, **2**, 363  
 Metoclopramide hydrochloride, **16**, 327  
 Metoprolol tartrate, **12**, 325  
 Metronidazole, **5**, 327  
 Minocycline, **6**, 323  
 Minoxidil, **17**, 185  
 Mitomycin C, **16**, 361  
 Mitoxantrone hydrochloride, **17**, 221  
 Morphine, **17**, 259  
 Moxalactam disodium, **13**, 305  
 Nabilone, **10**, 499  
 Nadolol, **9**, 455; **10**, 732  
 Nalidixic acid, **8**, 371  
 Naloxone hydrochloride, **14**, 453  
 Nalorphine hydrobromide, **18**, 195  
 Natamycin, **10**, 513  
 Neomycin, **8**, 399  
 Neostigmine, **16**, 403  
 Nifedipine, **18**, 221  
 Nitrazepam, **9**, 487  
 Nitrofurantoin, **5**, 345  
 Nitroglycerin, **9**, 519  
 Norethindrone, **4**, 268  
 Norgestrel, **4**, 294  
 Nortriptyline hydrochloride, **1**, 233; **2**, 573  
 Noscapine, **11**, 407  
 Nystatin, **6**, 341  
 Oxazepam, **3**, 441  
 Oxyphenbutazone, **13**, 333  
 Oxytocin, **10**, 563  
 Papaverine hydrochloride, **17**, 367  
 Penicillamine, **10**, 601  
 Penicillin-G benzothine, **11**, 463  
 Penicillin G, potassium, **15**, 427  
 Penicillin-V, **1**, 249; **17**, 677  
 Pentazocine, **13**, 361  
 Phenazopyridine hydrochloride, **3**, 465  
 Phenelzine sulfate, **2**, 383  
 Phenformin hydrochloride, **4**, 319; **5**, 429  
 Phenobarbital, **7**, 359  
 Phenoxymethyl penicillin potassium, **1**, 249  
 Phenylbutazone, **11**, 483  
 Phenylephrine hydrochloride, **3**, 483  
 Phenylpropanolamine hydrochloride, **12**, 357; **13**, 771  
 Phenytoin, **13**, 417  
 Physostigmine salicylate, **18**, 289  
 Phytonadione, **17**, 449  
 Pilocarpine, **12**, 385  
 Piperazine estrone sulfate, **5**, 375  
 Pirenzepine dihydrochloride, **16**, 445  
 Piroxicam, **15**, 509  
 Pralidoxine chloride, **17**, 533  
 Prazosin hydrochloride, **18**, 361  
 Primidone, **2**, 409; **17**, 749  
 Probenecid, **10**, 639  
 Procainamide hydrochloride, **4**, 333  
 Procarbazine hydrochloride, **5**, 403  
 Promethazine hydrochloride, **5**, 429  
 Proparacaine hydrochloride, **6**, 423  
 Propiomazine hydrochloride, **2**, 439  
 Propoxyphene hydrochloride, **1**, 301; **4**, 520; **6**, 598  
 Propylthiouracil, **6**, 457  
 Pseudoephedrine hydrochloride, **8**, 489  
 Pyrazinamide, **12**, 433  
 Pyridoxine hydrochloride, **13**, 447  
 Pyrimethamine, **12**, 463  
 Quinidine sulfate, **12**, 483  
 Quinine hydrochloride, **12**, 547  
 Ranitidine, **15**, 533  
 Reserpine, **4**, 384; **5**, 557; **13**, 737  
 Rifampin, **5**, 467  
 Rutin, **12**, 623  
 Saccharin, **13**, 487  
 Salbutamol, **10**, 665  
 Salicylamide, **13**, 521  
 Secobarbital sodium, **1**, 343  
 Silver sulfadiazine, **13**, 553  
 Sodium nitroprusside, **6**, 487; **15**, 781  
 Spironolactone, **4**, 431; **18**, 641  
 Streptomycin, **16**, 507  
 Strychnine, **15**, 563  
 Succinylcholine chloride, **10**, 691  
 Sulfadiazine, **11**, 523  
 Sulfadoxine, **17**, 571  
 Sulfamethazine, **7**, 401  
 Sulfamethoxazole, **2**, 467; **4**, 521  
 Sulfasalazine, **5**, 515  
 Sulfisoxazole, **2**, 487  
 Sulindac, **13**, 573  
 Sulphamerazine, **6**, 515  
 Sulpiride, **17**, 607  
 Terpin hydrate, **14**, 273  
 Testolactone, **5**, 533

Testosterone enanthate, <b>4</b> , 452	Thiothixene, <b>18</b> , 527
Tetracaine hydrochloride, <b>18</b> , 379	Timolol maleate, <b>16</b> , 641
Tetracycline hydrochloride, <b>13</b> , 597	Tolbutamide, <b>3</b> , 513; <b>5</b> , 557; <b>13</b> , 719
Theophylline, <b>4</b> , 466	Trazodone hydrochloride, <b>16</b> , 693
Thiabendazole, <b>16</b> , 611	Triamcinolone, <b>1</b> , 367; <b>2</b> , 571; <b>4</b> , 521, 524; <b>11</b> , 593
Thiamine hydrochloride, <b>18</b> , 413	Triamcinolone acetonide, <b>1</b> , 397, 416; <b>2</b> , 571; <b>4</b> , 521; <b>7</b> , 501; <b>11</b> , 615
Thioridazine and Thioridazine hydrochloride, <b>18</b> , 459	Triamcinolone diacetate, <b>1</b> , 423; <b>11</b> , 651
Thiostrepton, <b>7</b> , 423	